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14. ABSTRACT
The goal of this project was to understand how cyclophilin A (CypA) regulates the prolactin receptor (PRLr) signaling. The data have demonstrated that CypA, serving as a molecular switch via its peptidyl-prolyl isomerase (PPI) activity, binds to and regulates the function of the PRLr. Furthermore, point mutagenesis analysis has implied that a single prolyl imide bond (Pro-334) within the X-Box motif of intracellular domain of the PRLr is most likely responsible for the observed effects of CypA on the PRLr signaling. Finally, we have conducted several proof-of-concept studies that may lead to novel anti-breast cancer agents: 1) an FDA-approved CypA inhibitor CsA abrogates the PRLr signaling and tumor growth in vitro and in vivo, and our institution has started a phase-I clinical trial of this drug in breast cancer patients; 2) RNAi-mediated stable depletion of endogenous CypA protein inhibits PRLr signaling and blocks breast cancer cell motility and invasion; 3) a synthetic peptide comprising the X-Box motif inhibits PRLr signaling and downstream signaling responses; 4) synthetic antibodies, which exhibit nanomolar affinities toward the CypA, were generated using antibody phage display technology. Taken together, although the determination of the crystal structures of CypA in complex with the PRLr and/or its proximal molecule Jak2 was unsuccessful because of difficulties in obtaining high-pure and properly-fold proteins expressed in E. coli, the data reported herein strongly support a role for CypA in the PRLr function and breast cancer, and such findings could ultimately lead to the development of novel CypA inhibitors for the treatment of human breast cancer.

15. SUBJECT TERMS
cyclophilin A, prolactin receptor, Jak2,cyclosporine A, RNAi, peptide inhibitor, synthetic antibodies, breast cancer

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Table of Contents

INTRODUCTION

The growth and progression of human breast cancer is regulated by several cell surface receptors, including the prolactin receptor (PRLr). These receptor-triggered signals directly contribute to prolactin (PRL) induced proliferation, survival, and motility of human breast cancer [1]. The major objective of this work is to understand how cyclophilin A (CypA) regulates PRLr signaling and to determine the effects of altered CypA expression and activity on the PRLr signaling and breast cancer phenotypes. We aim to accomplish this through a multidisciplinary approach that combined cellular biological, biophysical structural and animal studies. There are two major hypotheses in this proposal: (1). CypA regulates PRLr signaling by altering structure of the PRLr complex via its intrinsic PPIase activity; (2). CypA level and activity contribute substantively to the biology of human breast cancer through its regulation of cell surface signaling, including that of the PRLr. We believe that the knowledge obtained from this work contribute significantly to a greater understanding of the mechanism of proximal PRLr signal transduction following ligand binding.

This final report summarizes the research conducted during the past three years. In Task 1, standard biochemical techniques, such as deletion mutagenesis and co-immunoprecipitation (co-IP) analysis, were used to determine the domains of the PRLr that interact with CypA. The data indicated that CypA binds to the PRLr through its X-Box motif [2]. In Task 2, a chaperone-assisted crystallography technology was used to determine the dynamic structure of CypA in complex with PRLr and its proximal molecule Jak2. We have purified recombinant CypA, the intracellular domain (ICD) of PRLr (PRLr-ICD), and the 4.1/ezrin/radixin/moesin domain (FERM) of Jak2 (Jak2-FERM) proteins expressed in *E. coli*. The major challenge we encountered is partial degradation of recombinant PRLr-ICD protein. In Task 3, the major approaches we used to manipulate CypA level and activity are CypA inhibitors (CsA and its analogs), siRNA-mediated suppression of CypA protein expression, peptide inhibitors that could compete CypA binding to the PRLr, and generation of synthetic antibodies that neutralize CypA activity. To test the functional effects of altering the structure of the PRLr complex by manipulating CypA expression and activity, we conducted PRL-induced cell signaling and gene expression studies, PRL-induced cell growth, motility, and invasion assays, and xenograft nude mice experiments. From a mechanistic perspective, our data demonstrated that CypA, which is implicated in the regulation of protein conformation, is necessary for the activation of PRL-induced signaling cascades. From a translational perspective, we have further demonstrated that siRNA-mediated CypA knockdown, a synthetic peptide comprising the X-Box motif, or CypA inhibitor (e.g. cyclosphorine A) abrogates PRLr signaling and breast cancer cell growth *in vitro* and/or *in vivo* [2]. In addition, by using a novel antibody phage display technology, we have successfully developed several synthetic antibodies, which exhibit nanomolar affinities toward CypA. Experiments are currently underway to test these synthetic CypA antibodies *in vitro* and *in vivo*. Given the vision of DoD-BCRP is to cure breast cancer, the successful development of such CypA inhibitors will open a new avenue for the development of novel anti-breast cancer agents, and ultimately benefit breast cancer patients [3; 4].

BODY

Three specific aims were proposed in this multidisciplinary postdoctoral award.
Specific Aim #1: To map the domains involved in the CypA-PRLr-Jak2 interactions.
Specific Aim #2: To determine the functional structure of the CypA-PRLr-Jak2 complex.
Specific Aim #3: To evaluate the functional effects of manipulating CypA level and activity on PRLr signaling and breast cancer phenotype *in vitro* and *in vivo*.

Statement of Work

The tasks to achieve the specific aims listed above are covered in the statement of work as outlined below.

Task 1: *In vitro* mapping of the CypA-PRLr-Jak2 interaction domains (Months 0-6).

a. Synthesis of Jak2 mutants and PRLr mutants by PCR-directed mutagenesis (Months 0-3).
b. Identification of the regions of the PRLr and Jak2 which bind to CypA by co-immunoprecipitation studies (Months 3-6).

Task 2: Determination of the functional structure of the CypA-PRLr-Jak2 complex (Months 6-36).

a. Co-crystallization of the CypA/PRLr/Jak2 interaction domains by a chaperone-assisted crystallography technology (Months 6-36).
b. Determination of the magnitude and nature of the conformational changes that are induced in the ICD of the PRLr and Jak2 by EPR spectroscopy analysis (Months 12-36).
c. Functional characterization of the putative proline residues that are the target of CypA activity by site-directed mutagenesis, co-immunoprecipitation, and *in vitro* cell culture models (Months 12-36).

Task 3: Assessment of the effects of manipulating CypA level and activity on PRLr signaling and breast cancer phenotype *in vitro* and *in vivo* (Months 12-36).

a. Design and synthesis of interaction-defective CypA mutants and constructs (Months 12-18).
b. Generation of stable transfectants that overexpress interaction-defective CypA mutant via a lentivirus-based delivery system, and that express the reduced level of CypA via a retriovirus-based siRNA knockdown system (Months 12-18).
c. *In vitro* testing of the effects of manipulating CypA level and activity on PRLr signaling. (Months 12-36).
d. *In vivo* testing of the effects of manipulating CypA level and activity on the biology of human breast cancer using nude mouse xenograft model. (Months 12-36).

Task 1. *In vitro* mapping of the CypA-PRLr-Jak2 interaction domains (Months 0-6).

To this end, standard biochemical techniques including deletion mutagenesis and co-IP analysis were utilized to determine the domains of the PRLr that interact with CypA. COOH-terminal PRLr truncation mutants were generated and tested for their ability when expressed to interact with CypA. Our published data [2] suggested that truncations of the PRLr membrane-proximal to the X-Box motif were incapable of interacting with CypA, revealing a contribution of the X-Box motif to the engagement of CypA. Taken together, our data suggests that the PRLr is associated with CypA through its X-Box motif [2]. A similar research strategy was used to map the interaction domains of Jak2 with CypA, and such binding sites on Jak2 remain to be determined.

Task 2: Determination of the functional structure of the CypA-PRLr-Jak2 complex (Months 6-36).

This task focuses on the elucidation of the structural basis for the CypA-PRLr-Jak2 complex using several biophysical techniques including X-ray crystallization, electron paramagnetic resonance (EPR) analysis and other biophysical approaches. In order to accomplish this aim, we must purify a large amount of high-pure and active recombinant proteins using the prokaryotic expression system for crystallization and EPR experiments.

PCR-amplified CypA DNA fragment for the expression of recombinant human CypA, was cloned into pMCSG19C prokaryotic vector using ligation-independent cloning method as described [5]. The cloned gene in pMCSG19C was expressed as a fusion protein with a N-terminal dual tag, which is ordered by maltose-binding protein (MBP), a TVMV protease cleave site, poly-histidine, and a TEV protease cleave site (left panel in Figure 1A). This prokaryotic expression system has several advantages over others. For example, fusion to MBP significantly enhances protein solubility in most cases. In addition, the MBP tag could be cleaved in cells co-expressing TVMV protease, such as *E. coli* BL21(DE3) (Novagen). In our experience, this system usually expresses foreign proteins at a high level, and the his_6-tag could also be removed by TEV protease *in vitro*. In brief, *E. coli* BL21(DE3) was transformed with the resulting construct DNA and cells were grown in LB medium supplemented with amplicilin (100 μg/ml). Expression of recombinant CypA protein was initiated by the addition of IPTG (0.1 mM) at OD_{600} of around 0.6-0.7. After overnight induction at 25°C, highly soluble 6His-tag recombinant CypA protein was purified on Ni-NTA resin under native conditions. In general, using this expression approach we were able to obtain up to 20 mg of recombinant proteins (>95% pure as estimated by SDS-PAGE analysis, about 18 kDa) per liter of bacterial culture (right panel in Figure 1A). The purified proteins were frozen at -80°C before they were used for experiments.

For the expression of recombinant human PRLr-ICD protein, DNA fragment encoding amino acids 259-349 of the intermediate form of the PRLr [6] was amplified by PCR and cloned into pGEX vector (GST Gene Fusion System, GE Healthcare life Sciences) (left panel in Figure 1B). The resulting construct was verified by DNA sequencing and used to transform *E. coli* BL21 (DE3) cells. Cultures were grown in LB medium with amplicilin (100 μg/ml) at 37°C until OD_{600} reach about 0.6-0.7. Expression of recombinant PRLr-ICD protein was induced by IPTG (0.1 mM) overnight at 18°C. Cells collected by centrifugation were resuspended in the lysis buffer (50 mM Tris HCL buffer (pH7.5) containing 200 mM NaCl, 10 mM MgSO4, 50 μg/ml Dnase and 1x protease inhibitor cocktail), and lysed by French pressure cell press method. Recombinant GST-PRLr-ICD protein was recovered from cell extracts on glutathione-superflow resin (Clontech). In order to mimic the predimerized PRLr in cells, we kept GST-PRLr-ICD fusion protein intact since GST or GST fusion proteins are known to dimerize. Using this expression system we were able to obtain up to 1 mg of recombinant proteins (>80% pure as estimated by SDS-PAGE analysis, about 40 kDa) per liter of bacterial culture (right panel in Figure 1B). The purified proteins were frozen at -80°C before they were used for experiments. The major challenge we encountered in the purification of recombinant GST-PRLr-ICD protein was partial protein degradation as indicated in the right panel of Figure 1B. In order to obtain high-pure protein, further work to improve methods for preventing the degradation of recombinant PRLr proteins is needed.

In order to express recombinant human Jak2-FERM protein, the gene fragment corresponding to residues 1-382 of human Jak2 was cloned into *E. coli* expression vector (pGEX vector) using standard PCR protocols (left panel in Figure 1C). Like human PRLr-ICD protein, this Jak2 FERM protein was expressed as a fusion protein with GST tag on its N-terminus. In briefly, *E. coli* BL21(DE3) was transformed with the resulting gene expression plasmid and cells were grown overnight at 25°C. Protein expression was induced by an addition of 0.1 mM IPTG, and purified from cell extracts on glutathione-superflow resin (Clontech) (right panel in Figure 1C). The purified proteins were frozen at -80°C before they were used for experiments.

Once having purified these proteins (CypA, PRLr-ICD and Jak2 FERM) to near homogeneity at a larger scale, we will proceed with crystallization and EPR experiments.

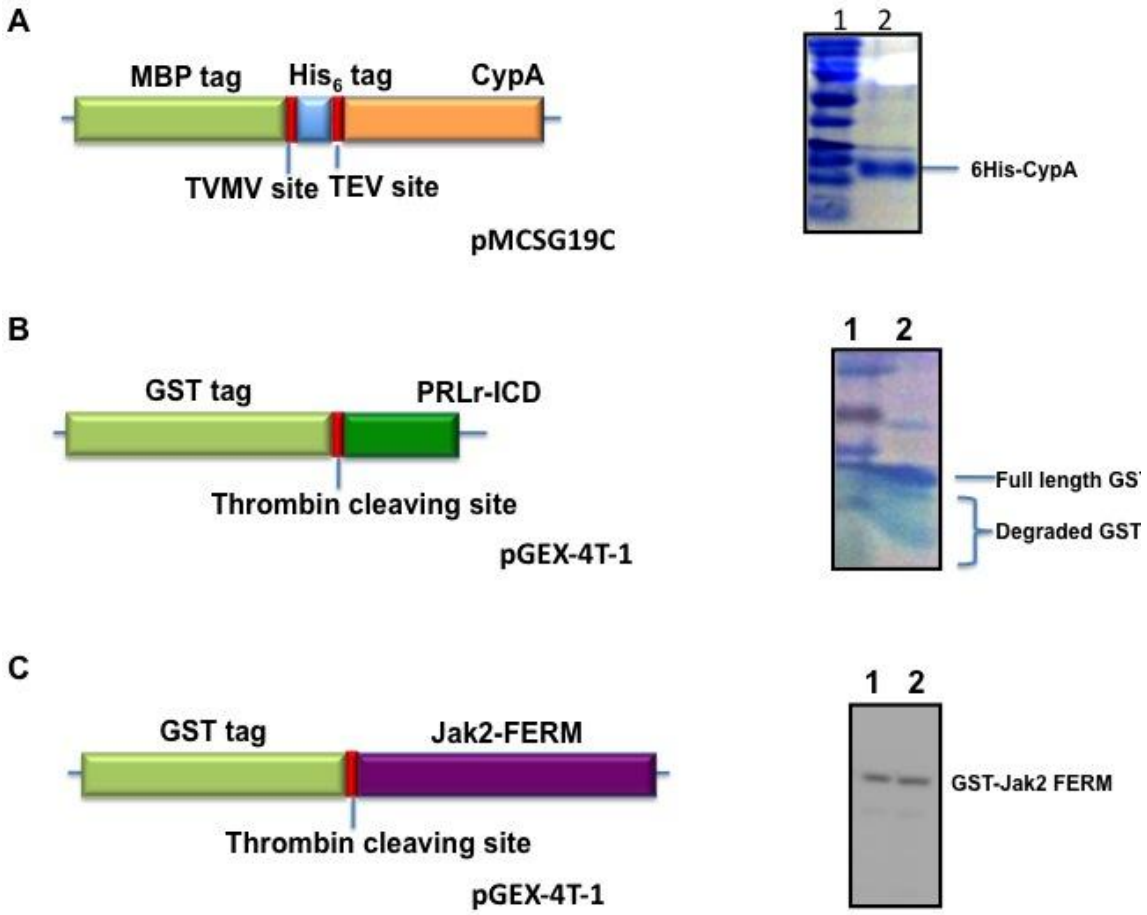

Figure 1. Expression and purification of human CypA, PRLr-ICD and Jak2 FERM proteins. (A) Expression of human CypA protein. Left panel shown is a schematic diagram of recombinant his-tagged CypA protein expressed in *E. coli*. Right panel shown is recombinant his-tagged CypA protein. Lane1: protein marker; lanes 2: recombinant CypA protein. The position of recombinant CypA protein is indicated with an arrow. (B) Expression of human PRLr-ICD domain (259-349) protein. Left panel shown is a schematic diagram of recombinant GST-tagged PRLr-ICD protein. Right panel shown is recombinant GST-tagged PRLr-ICD protein. Lane1: protein marker; lanes 2: recombinant GST-tagged PRLr-ICD protein. The position of recombinant GST-tagged PRLr-ICD protein and its partial protein degradation are indicated with symbols. (C) Expression of human Jak2 FERM protein. Left panel shown is a schematic diagram of recombinant GST-tagged Jak2 FERM protein expressed in *E. coli*. Right panel shown is recombinant GST-tagged Jak2 FERM protein.

Task 3: Assessment of the effects of manipulating CypA levels and PPIase activity on PRLr signaling and breast cancer phenotype *in vitro* **and** *in vivo* **(Months 12-36).**

Our data suggested that CypA, serving as a molecular switch via its peptidyl-prolyl isomerase (PPI) activity, binds to the PRLr and regulates its functions in breast cancer [2]. The major approaches we used to manipulate CypA level and activity are CypA inhibitors (CsA and its analogs), siRNA-mediated suppression of CypA protein expression, peptide inhibitors that could compete CypA binding to the PRLr, and generation of synthetic antibodies that neutralize CypA activity.

First of all, using CypA inhibitors, we have demonstrated that CsA as we previously published [2] and its non-immunosuppressive analogs including CsD and CsH significantly abrogated PRLr signaling and PRL-induced gene expression, inhibited cell growth, motility and invasion *in vitro*, and blocked tumor growth *in vivo* (summarized in Table 1). Thus, these data suggested that the development of novel CypA inhibitors represents a useful therapeutic strategy in the treatment of breast cancer patients.

CypA inhibitors	anchorage-dependent growth	anchorage-independent growth	Cell motility and invasion in vitro	PRL signaling	PRL gene expression	Tumor growth in vivo
CsA	+	+	+	+	+	+
CsD	+	+	ND	+	ND	ND
CsH	+	+	ND	+	ND	ND

+: significantly inhibit; ND: not determined

Table 1. The inhibition of CypA activity by CypA inhibitors (CsA, CsD and CsH) is associated with abrogation of PRLr signaling and tumor growth *in vitro* and *in vivo*. CsA is an FDA-approved immunosuppressive drug, and CsD/CsH are non-immunosuppressive chemically modified derivatives of CsA. Using *in vitro* breast cancer cell models (ER+: T47D and MCF7; ER-: MDA231) and/or *in vivo* nude mouse xenograft models, we demonstrated that CsA and its analogs have anti-breast cancer efficacy. *In vitro*, CsA and its analogs (CsD and CsH) inhibit PRL signaling (Jak2, Stat5, Erk and Akt) and PRL-induced gene expression (CISH and Cyclin D1), suppress anchorage-dependent and independent cell growth, and block breast cancer cell motility and invasion. *In vivo*, we further demonstrated that CsA significantly enhances central necrosis of primary tumors and blocks tumor metastasis.

Secondly, we have identified the X-Box motif of the PRLr as an essential mediator for the interaction of the PRLr with CypA [2]. As consequence of this finding, a peptide inhibitor was developed to specifically block the activation of the PRLr signaling and downstream signaling responses. We synthesized the X-Box peptide and examined its effects on PRL signaling in T47D (Figure 2A). Our data demonstrated that the X-Box peptide but not control peptides (either cell-permeable peptide or P334A peptide) was able to significantly block PRLr signaling and reduce PRL-induced gene expression from both CISH luciferase reporter and endogenous CISH (Figure 2B-D). Taken together, these data suggest that the X-Box peptide appears to be a specific inhibitor of PRLr signaling.

Thirdly, we provided evidence that elimination of endogenous CypA protein by using RNA interference technology resulted in a significant inhibition of Jak2, Stat5, ERK1/2 and Akt activation in breast cancer cells (Figure 3A). More importantly, siRNA-mediated knockdown of CypA can block motility and invasion of breast cancer cells *in vitro* (Figure 3B-C). Thus, suppression of CypA expression using small interfering RNA may hold a potential as a therapeutic strategy for breast cancer. The concept of the use of RNA interference to suppress expression of oncogenes in breast cancer is encouraged by a recent study that nanoparticle-delivered siRNA successfully turns off

expression of ribonucleotide reductase (RRM2) gene in human tumors [7].

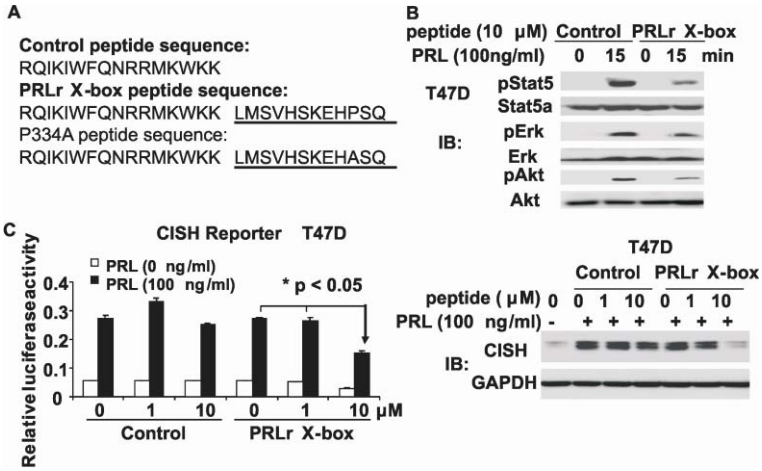

Figure 2. The X-Box peptie inhibits PRLr signaling. (A) Sequences of controls and the X-Box peptides. Control peptide is the antenapedia homeodomain, a cell-permeable peptide. (B) The X-Box peptide blocks the activation of PRLr signaling. T47D cells were starved overnight and pre-treated with 10 μM of control or X-Box peptide for 1 hour as indicated. Cells were stimulated with PRL (100 ng/ml) for 15 mins. Blots were probed with the indicated antibodies. (C) The X-Box peptide inhibits PRL-induced gene expression from both CISH luciferase reporter and endogenous CISH. T47D cells were transfected with CISH luciferase reporter. Cells were starved overnight and pre-treated with 0, 1, and 10 μM of control or the X-Box peptide for 1 hour as indicated. After 24h PRL (100 ng/ml) stimulation, cell lysates were analyzed for both luciferase activity and CISH protein. The results are representative of at least two independent experiments.

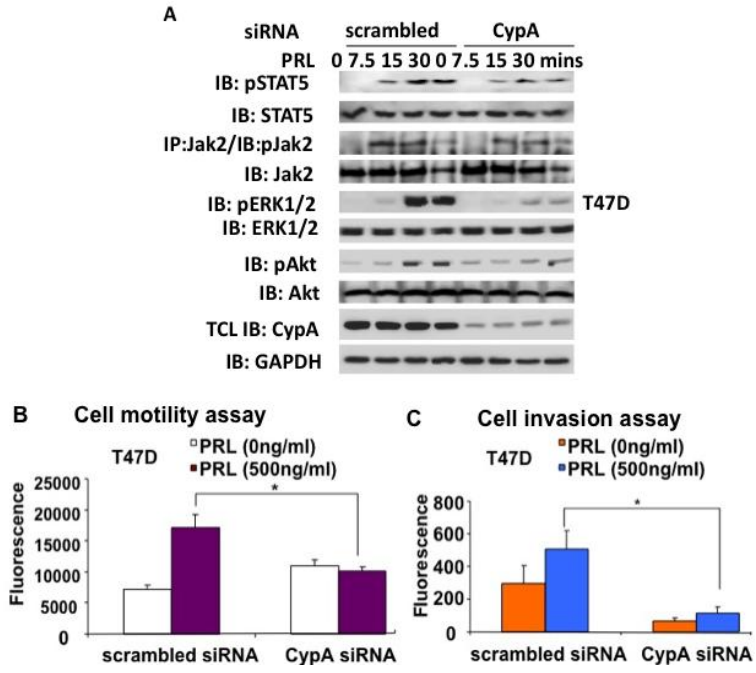

Figure 3. siRNA-mediated knockdown of CypA inhibits PRL-induced signaling and gene expression, and blocks human breast cancer cell motility and invasion. (A) Stable depletion of CypA inhibits PRLr signaling. Stable pools of T47D transfectants expressing scrambled or CypA siRNA were stimulated with PRL (100 ng/ml) at indicated times, harvested, and lysates were subject to immunoblot analysis as indicated. (B-C) Stable depletion of CypA blocks motility and invasion of breast cancer cells. Stable transfectants of T47D cells with scrambled siRNA and CypA siRNA were allowed to migrate or invade toward 3% fetal bovine serum for 24 hours. The number of cells migrating or invading to the lower surface of the membrane was quantified by CyQuant (Invitrogen).

Lastly, using an antibody phage display technology, we have successfully generated 18 synthetic antibodies targeted to CypA. Antibody-based therapies are highly advantageous because they provide specificity, thus minimizing toxicity. By using the most appropriate delivery techniques, we can deliver these synthetic antibodies into cancer cells, and stop cancer cell growth. The production of functional synthetic antibodies involves immobilization of target proteins, phase display library screening, and functional analysis of selected antigen-binding fragments (FABs) (Figure 4A) [8]. In brief, recombinant his-tag CypA proteins were produced and purified as described in Task 2. Target proteins were then biotinylated using EZ-Link NHS-SS-PEG4-Biotin kit (Pierce), and the resulting proteins were immobilized on streptavidin-coated paramagnetic beads. The immobilized target proteins were mixed with the YSG FAB phage library (Genentech, Inc.) to capture target-specific phage particles. After three to four rounds of library sorting and amplification, individual bound phages were randomly selected for competitive phage enzyme-linked immunosorbent assay (ELISA) in the present or absence of competitors (soluble native CypA proteins or CsA). Positive clones were sequenced and the FABs with unique CDRs were identified. As shown in Figure 4B, phage display library screening results in the generation of total 18 CypA-specific FABs including CsA sensitive (#1-9) and non CsA-sensitive (#10-18). A free protein form of FABs other than FAB-p3 fusion protein was achieved by the introduction of a stop codon between the FAB heavy chain and the phage p3 coat protein using kunkel mutagenesis. The 55244 *E. coli* strain (Genentch, Inc) was transformed with the phagemid DNA, and cultures were grown in the CRAP medium supplemented with amplicilin (100 μg/ml) for 24 hours at 30°C. The FAB proteins were purified with protein A resin (Figure 4C) and subjected to binding affinity analysis. Based on our preliminary surface plasmon resonance (SPR) analysis, several FABs have exhibited binding affinity within a low nanomolar range, and are good candidates for further studies (Table 2). Experiments are currently underway to test these potential synthetic antibodies *in vitro* and *in vivo*.

FABs	3	4	5	8	10	14	16	18
Kd (nM)	3	2	7	12	2	12	4	0.9

Table 2. Preliminary SPR analysis of binding affinity of selected FABs. Purified FABs were injected over sensor chip NTA where 6His-tag CypA was immobilized on the surface. The equilibrium constant Kd was used to measure the binding affinity of two components.

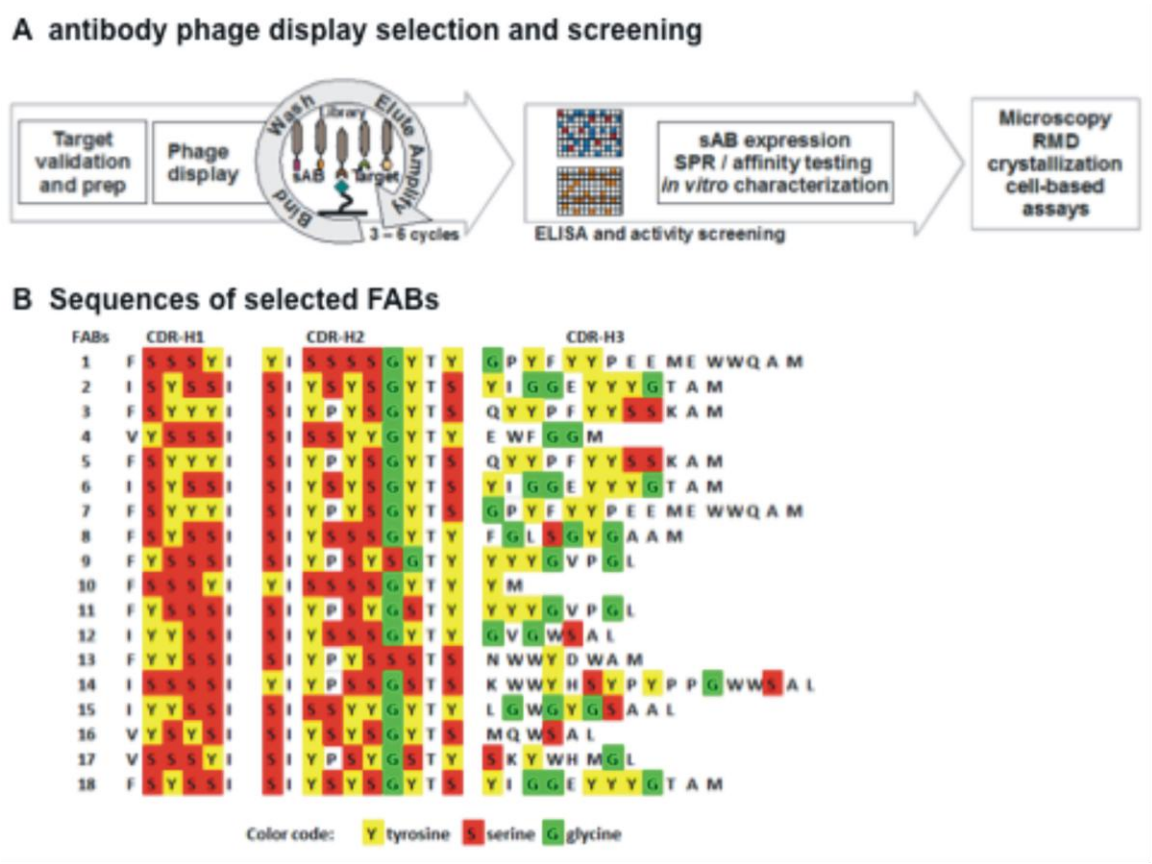

Figure 4. Generation of synthetic antibodies targeted to the CypA. (A) A schematic diagram of synthetic antibody production, which involves target protein immobilization, phase display library screening, and structural and functional analysis of selected FABs. (B) Sequences of selected FABs. Tyrosine (Y), Serine (S) and Glycine (G) are randomized residues within complementarity determining region (CDR) H1, H2 and H3. In order to show their abundance in the sequence, these residues are highlighted by yellow, red and green, respectively. (C) Expression and purification of selected FABs. Through the introduction of a stop codon between the FAB heavy chain and the phage p3 coat protein using Kunkel mutagenesis, a soluble form of FABs was obtained. The resulting phagemid DNAs were used to transform *E. coli* 55244 and the FAB proteins were expressed under the PhoA alkaline phosphatase promoter in the CRAP medium at 30 °C for 24 hours. The FAB proteins were purified on protein A resin. Lane 1-18: recombinant FAB proteins (#1-18) (>90% pure as estimated by coomassie blue-stained SDS-PAGE gel).

KEY RESEARCH ACCOMPLISHMENTS

- Identifying of the X-Box motif of intracellular domain of the PRLr that interacts with CypA
- Cloning of human CypA, PRLr-ICD, and Jak2-FERM into prokaryotic expression vectors.
- Expressing and purifying of human CypA, PRLr-ICD and Jak2-FERM proteins in *E. coli* BL21(DE3) cells.
- *In vitro* and *in vivo* CsA treatment of human breast cancer
- Starting of a phase I clinical trial of CsA in breast cancer patients
- Developing of a peptide inhibitor that blocks PRLr signaling.
- siRNA-mediated stable depletion of endogenous CypA protein in breast cancer cells.
- Generation of synthetic antibodies against CypA using an antibody phage display technology.

REPORTABLE OUTCOMES

Journal papers

Zheng, J., J. E. Koblinski, L. V. Dutson, Y. B. Feeney, and C. V. Clevenger. 2008. Prolyl isomerase cyclophilin A regulation of Janus-activated kinase 2 and the progression of human breast cancer. Cancer Research 68:7769-7778.

Clevenger, C.V., S.L. Gadd, and J. Zheng. 2009. New mechanisms for PRLr action in breast cancer. Trends in Endocrinology and Metabolism. Trends in Endocrinology and Metabolism. 20 (5): 223-9.

Conference abstracts and posters

Zheng J., A. Kossiakoff, and C. Clevenger. “Multidisciplinary Analysis of Cyclophilin A/Prolactin Receptor Complex Function in Human Breast Cancer”. Dod The Leading Innovation and Knowledge Sharing (LINKS) Meeting, Vienna, VA, February 9 – 10, 2009. Poster

Zheng J., A. Paduch M., Harrington K., Kossiakoff A., and Clevenger C. “Multidisciplinary Analysis of Cyclophilin A/Prolactin Receptor Complex Function in Human Breast Cancer”. DOD The Leading Innovation and Knowledge Sharing (LINKS) Meeting, Vienna, VA, February 16 – 17, 2010. Poster

Zheng J., A. Paduch M., Harrington K., Kossiakoff A., and Clevenger C. “Multidisciplinary Analysis of Cyclophilin A/Prolactin Receptor Complex Function in Human Breast Cancer”. DOD The Leading Innovation and Knowledge Sharing (LINKS) Meeting, Vienna, VA, February 16 – 17, 2011. Poster

CONCLUSIONS

In summary, we have discovered that CypA, serving as a molecular switch via its peptidyl-prolyl cis-trans isomerase activity, binds to and regulates the function of the PRLr. A single prolyl imide bond (Pro-334) within the intracellular domain of the PRLr is most likely responsible for the observed effects of CypA on PRLr signaling because a synthetic peptide comprising this motif inhibits PRLr signaling, while mutation of Pro-334 to alanine abolishes its effects. From a translational perspective, we have shown that the inhibitor of CypA, cyclosporine A (CsA), impairs this interaction, inhibits PRLr signaling, and stops breast cancer cell growth and tumor metastasis. It is singularly on the basis of these spectacular findings, that our institution has started a clinical trial of this drug in breast cancer patients. Furthermore, we have successfully developed several synthetic antibodies targeted to CypA using a novel antibody phage display technology. Using synthetic antibodies to inhibit CypA function and PRLr signaling in breast cancer is a very original and innovative approach and may lead to novel CypA inhibitors that are more efficient and cost-effective. In the event that we obtain high-pure and properly-folded PRLr and Jak2-FERM, we will be ready to solve the crystal structure of CypA in complex with PRLr and/or its proximal molecule Jak2. Future understanding the structural basis of the CypA-PRLr-Jak2 interactions should allow us to unravel the mechanisms of how CypA modulates the PRLr function at the atomic level.

REFERENCES

[1]C.V. Clevenger, P.A. Furth, S.E. Hankinson, L.A. Schuler, The role of prolactin in mammary carcinoma. Endocr Rev 24 (2003) 1-27.

[2]J. Zheng, J.E. Koblinski, L.V. Dutson, Y.B. Feeney, C.V. Clevenger, Prolyl isomerase cyclophilin A regulation of Janus-activated kinase 2 and the progression of human breast cancer. Cancer Res 68 (2008) 7769-7778.

[3]C.V. Clevenger, S.L. Gadd, J. Zheng, New mechanisms for PRLr action in breast cancer. Trends Endocrinol Metab 20 (2009) 223-229.

[4]C.V. Clevenger, J. Zheng, E.M. Jablonski, T.L. Galbaugh, F. Fang, From bench to bedside: future potential for the translation of prolactin inhibitors as breast cancer therapeutics. J Mammary Gland Biol Neoplasia 13 (2008) 147-156.

[5]M.I. Donnelly, M. Zhou, C.S. Millard, S. Clancy, L. Stols, W.H. Eschenfeldt, F.R. Collart, A. Joachimiak, An expression vector tailored for large-scale, high-throughput purification of recombinant proteins. Protein Expr Purif 47 (2006) 446-454.

[6]J.B. Kline, H. Roehrs, C.V. Clevenger, Functional characterization of the intermediate isoform of the human prolactin receptor. J Biol Chem 274 (1999) 35461-35468.

[7]M.E. Davis, J.E. Zuckerman, C.H. Choi, D. Seligson, A. Tolcher, C.A. Alabi, Y. Yen, J.D. Heidel, A. Ribas, Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature (2010).

[8]F.A. Fellouse, C. Wiesmann, S.S. Sidhu, Synthetic antibodies from a four-amino-acid code: a dominant role for tyrosine in antigen recognition. Proc Natl Acad Sci U S A 101 (2004) 12467-12472.

APPENDICES

A copy of the published paper: Zheng, J., J. E. Koblinski, L. V. Dutson, Y. B. Feeney, and C. V. Clevenger. 2008. Prolyl isomerase cyclophilin A regulation of Janus-activated kinase 2 and the progression of human breast cancer. Cancer Research 68:7769-7778.

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Prolyl Isomerase Cyclophilin A Regulation of Janus-Activated Kinase 2 and the Progression of Human Breast Cancer

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Abstract

The activation of the Janus-activated kinase 2 (Jak2) tyrosine kinase following ligand binding has remained incompletely characterized at the mechanistic level. We report that the peptidyl-prolyl isomerase (PPI) cyclophilin A (CypA), which is implicated in the regulation of protein conformation, is necessary for the prolactin (PRL)-induced activation of Jak2 and the progression of human breast cancer. A direct correlation was observed between the levels or activity of CypA and the extent of PRL-induced signaling and gene expression. Loss of PRLr-CypA binding, following treatment with the PPI inhibitor cyclosporine A (CsA), or overexpression of a dominant-negative PRLr mutant (P334A) resulted in a loss of PRLr/Jak2–mediated signaling. *In vitro*, CsA treatment of breast cancer cells inhibited their growth, motility, invasion, and soft agar colony formation. *In vivo*, CsA treatment of nude mice xenografted with breast cancer cells induced tumor necrosis and completely inhibited metastasis. These studies reveal that a CypA-mediated conformational change within the PRLr/Jak2 complex is required for PRL-induced transduction and function and indicate that the inhibition of prolyl isomerases may be a novel therapeutic strategy in the treatment of human breast cancer. [Cancer Res 2008;68(19):7769–78]

Introduction

Peptidyl-prolyl isomerases (PPI) are a family of enzymes that catalyze the *cis-trans* interconversion of imide bonds of proline residues (1). One member of this family, cyclophilin A (CypA), was initially identified as the primary cytosolic receptor of the immunosuppressive drug cyclosporine A (CsA; ref. 2). The immunosuppressive activity of CsA is thought to be mediated by the engagement of calcineurin by the CsA-CypA complex (3), an observation supported by the finding that CypA knockout mice are resistant to immunosuppression by CsA (4). As a PPI, CypA has been found to assist in protein folding *in vivo* (5). Several lines of research, however, have revealed that CypA and other PPIs may function as molecular signaling "switches" (6). Indeed, it has been shown that PPI activity promotes viral replication and infection of HIV (7), modulates Itk and Crk signaling (8, 9), regulates the pore opening of a neurotransmitter-gated ion channel (10), functions as a molecular timer for phage infection (11), and controls transcription factor activity (12).

Additional data also suggest that CypA may contribute to the pathology of human malignancy. CypA is overexpressed in a large number of lung (13), pancreatic (14), and oral squamous cancer cells and tissues (15). Significantly, in a case cohort of 25,000 women who had received CsA as therapy for renal and cardiac allografts, a reduction in the incidence of breast cancer of up to 50% in the CsA-treated group was noted during a 10-year follow-up period. Indeed, in their first year of therapy, the CsA-treated cohort showed a 90% reduction in the incidence of breast cancer (16). Taken together, these data suggest that CypA may play an important role in tumorigenesis and serve as a target for PPI inhibitors in the oncologic setting.

The important role of the hormone prolactin (PRL) in the development and progression of breast cancer has been supported by epidemiologic studies and transgenic model studies (17–19). The effects of PRL on normal and malignant breast tissues are mediated by the prolactin receptor (PRLr), a member of the cytokine receptor superfamily and relative of the growth hormone receptor. Both the growth hormone receptor and the PRLr are predimerized; following ligand binding, it has been reported that the growth hormone receptor and PRLr may undergo conformational change (20, 21), enabling the rapid activation of the Janus-activated kinase 2 (Jak2) tyrosine kinase, which results in PRLr/growth hormone receptor phosphorylation and the activation of several signaling cascades including the Jak2/signal transducer and activator of transcription-5 (Stat5), Ras/mitogen-activated protein kinase, and Nek3/Vav2/Rac1 pathways (17, 22). The triggering of these signaling pathways summates in the activation of multiple gene loci, including cyclin D1 (23) and the cytokine inducible SH2-containing protein (CISH; ref. 24). Like other cytokine receptors that associate with Jak2, the activation of many of these downstream signaling pathways is dependent on the initial activation of Jak2. However, the molecular mechanism following ligand binding that triggers a conformational change in the Jak2-receptor complex enabling Jak2 activation has remained elusive. Given the important role that the PRLr, growth hormone receptor, and other cytokine receptors play in normal and pathologic physiology, an understanding of this phenomenon is highly relevant.

Our laboratory has previously reported on a role for cyclophilin family members in the regulation of the PRLr-mediated signaling. Triggered by PRL, cyclophilin B potentiated Stat5 function by inducing the release of the Stat repressor, PIAS3 (12, 25). Here, we report that the PPI activity of CypA contributes to the proximal activation of Jak2 and other PRLr-associated signaling pathways following binding of ligand to the PRLr as a receptor-associated conformational switch. Finally, our findings reveal that ablation of CypA activity by several approaches, including the use of the Food

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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and Drug Administration–approved drug CsA, inhibits the *in vitro* and *in vivo* outgrowth of human breast cancer.

Materials and Methods

Cell culture and reagents. The human breast cancer cell lines T47D, MCF7, and MDA-231 were maintained in recommended conditions (26). The mouse pre-adipocyte cell line 3T3F442A, kindly provided by Dr. Stuart J. Frank (Department of Medicine, University of Alabama, Birmingham, AL), was maintained in DMEM supplemented with 10% calf serum. CsA (Gengraf) was purchased from Abbott Laboratories. Recombinant human PRL was obtained from Dr. Michael Hodsdon (Department of Pathology, Yale University, New Haven, CT), and recombinant human growth hormone was from Dr. Stuart J. Frank. CyQuant (Invitrogen) and QuickChange (Stratagene) were used as directed. Antibodies used in these studies were obtained from Santa Cruz (cyclophilin A and CISH), Invitrogen (Stat5a, pStat5a, glyceraldehyde-3-phosphate dehydrogenase, V5, cyclin D1, and PRLr-ECD), and Cell Signaling [Jak2, pJak2, extracellular signal–regulated kinase (Erk)-1/2, pErk1/2, Akt, and pAkt].

Immunoblotting and immunoprecipitation. Immunoblotting and immunoprecipitation were done as previously described (26). Target proteins were visualized by enhanced chemiluminescence (GE Healthcare), and images were captured using Fujifilm LAS-3000 system. The band intensities were quantified by densitometry using ImageQuant and normalized to those of their respective control bands. Data were expressed as fold changes compared with an appropriate control.

Plasmids, transfection, and retrovirus production. The PRLr, CypA constructs, Stat5-responsive luteinizing hormone response element (LHRE), and CISH reporters were previously described (26, 27). A set of COOH-terminal deletion constructs of the PRLr were assembled into the pTracer vector. A V5-tag was fused to the 3′ end of all deleted constructs. All point mutations were generated using the QuickChange kit. The pSilencer 5.1 carrying a DNA insert encoding a CypA siRNA or a scrambled control siRNA was purchased from Ambion.

The PRL-induced, luciferase-linked gene reporters used here contained either a synthetic LHRE reporter or the endogenous promoters from the *CISH* gene. For luciferase assay, cells were transiently transfected with the LHRE and CISH reporters, in addition to either the empty vector (control)

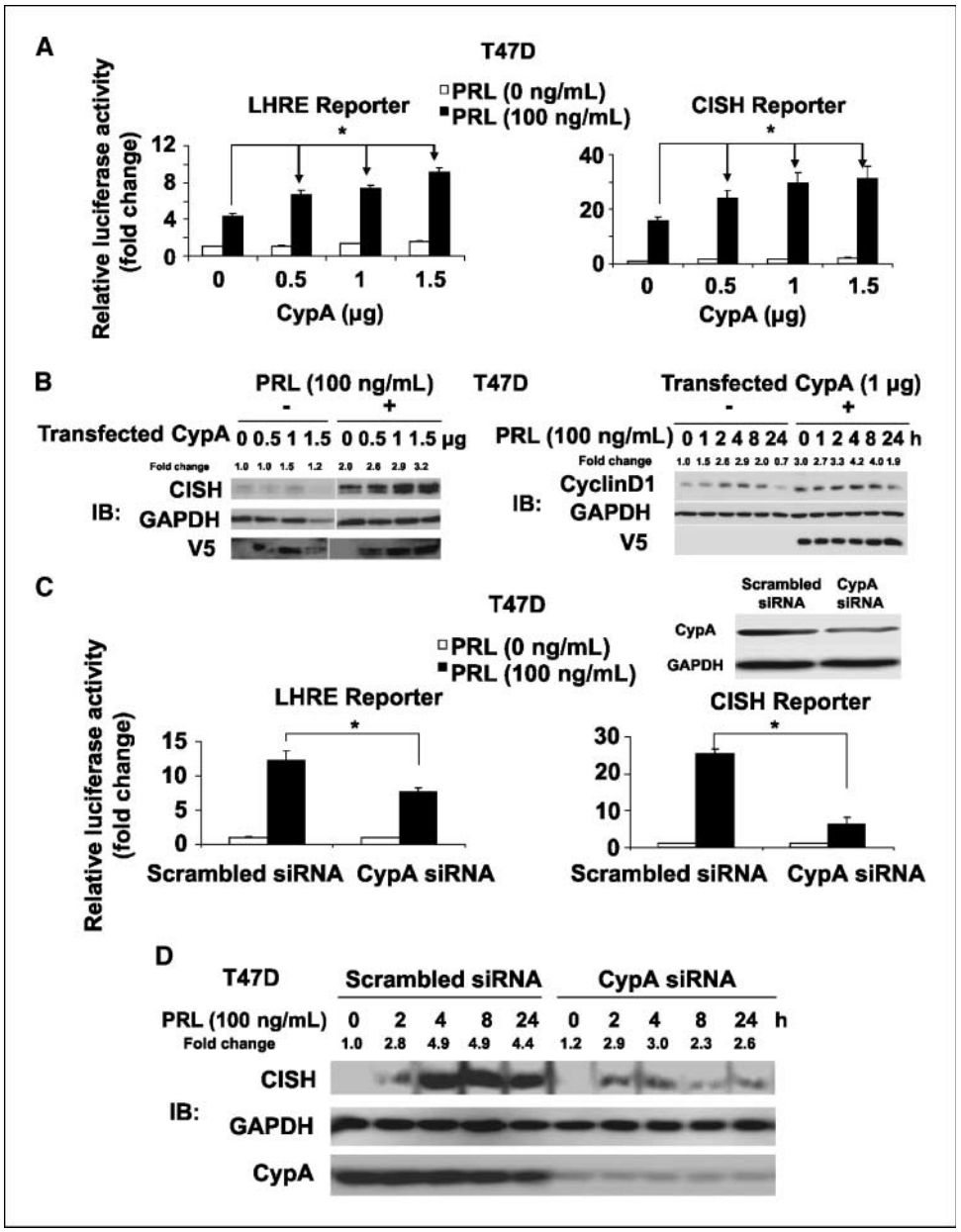

Figure 1. Modulation of CypA levels regulates PRL-induced gene reporter and endogenous gene expression. *A,* CypA overexpression enhances PRL-induced gene expression from luciferase reporters. T47D cells were cotransfected with LHRE or CISH reporters with increasing amount of CypA overexpression construct as indicated. Cells were treated with PRL (100 ng/mL) in defined medium for 24 h. *Columns,* mean of at least two independent experiments; *bars,* SE. *, $P < 0.05$ (*t* test). *B,* CypA overexpression enhances PRL-induced endogenous gene expression of CISH and cyclin D1 proteins in T47D cells. The expression of transfected CypA was verified by Western blot analysis of total cell lysates with an anti-V5 antibody. *C,* siRNA-mediated knockdown of CypA results in reduced PRL-induced reporter gene expression. T47D cells were transfected with CypA siRNA or scrambled control siRNA and the indicated luciferase reporters. Following 24-h stimulation with PRL (100 ng/mL), transfectants were lysed and assayed for luciferase activity. Knockdown of CypA by siRNA was confirmed by Western blot analysis (*top right*). *Columns,* mean of at least two independent experiments; *bars,* SE. *, $P < 0.05$ (*t* test). *D,* stable depletion of CypA represses endogenous *CISH* gene expression in T47D cells. Stable transfectants of T47D cells expressing either scrambled siRNA or CypA siRNA were stimulated with PRL (100 ng/mL) for 24 h. Suppression of endogenous, PRL-induced CISH expression by CypA knockdown was detected by Western blot analysis.

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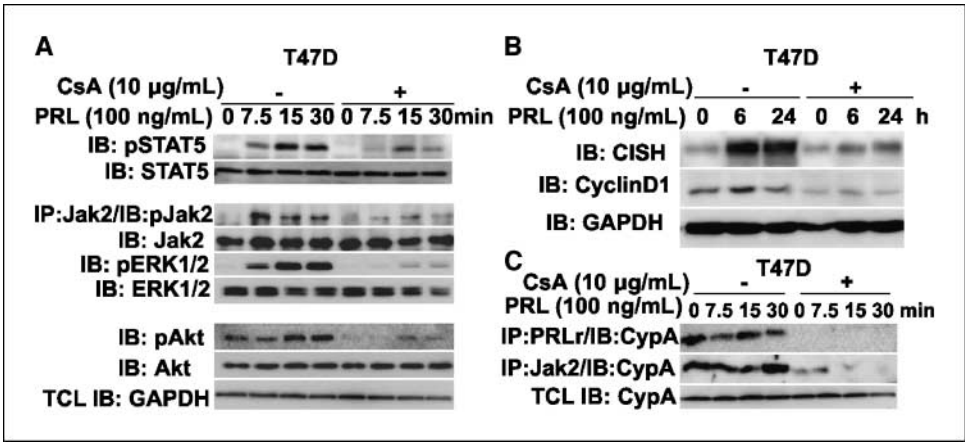

Figure 2. Ablation of CypA activity by CsA suppresses the PRLr-associated signaling, reduces PRL-induced gene expression, and inhibits the association of CypA with the PRLr and Jak2. *A,* CsA inhibits PRLr signaling. T47D cells in defined medium were pretreated with CsA for 4 to 6 h. Cells were stimulated with PRL (100 ng/mL) at the indicated times. Blots were probed with the indicated antibodies. *TCL,* total cell lysates; *IP,* immunoprecipitation. *B,* CsA treatment of T47D cells results in reduced PRL-induced gene expression. T47D cells treated without or with CsA for 6 or 24 h were assessed for CISH and cyclin D1 protein expression by Western blot analyses. *C,* CsA inhibits the association of CypA with the PRLr and Jak2. T47D cells in defined medium were treated as described above. Cell lysates were immunoprecipitated with anti-PRLr-ECD or anti-Jak2 antibody and immunoblotted with anti-CypA antibody. Representative of three independent experiments.

or the indicated plasmid. Cells were arrested for 24 h posttransfection and then unstimulated or treated with the indicated amount of PRL. Twenty-four hours after PRL stimulation, cells were lysed and extracts were assayed using Victor3 Multilabel Counter (Perkin-Elmer).

The association of PRLr P334A or PRLr deletion mutants with wild-type CypA, the association of Cyp-PPI mutants with wild-type PRLr, and the Stat5 activation by P334A mutant were determined in 293FT cells transfected with the indicated plasmids. The effects of wild-type CypA, Cyp-PPI, and PRLr P334A overexpression on endogenous gene expression were assessed in T47D cells transfected with the increased amounts of plasmids as indicated. In all transfection experiments, the total amount of transfected plasmid was equalized with empty vector.

For production of recombinant retroviruses, PT67 cells were transfected with pSilencer 5.1 Retro-CypA siRNA or pSilencer 5.1 Retro-scrambled siRNA, and viruses were harvested 48 h posttransfection. To generate stable knockdown cells, T47D cells were infected with the retroviral supernatants, and stable colonies were selected with 2 μg/mL puromycin.

Cell growth assay. Cells were plated the day before CsA treatment in 24-well plates at 1×10^4 per well. Cells were incubated in complete medium with various concentrations of CsA as indicated for an additional 6 d, and viable cells were counted with a hemocytometer every other day by the trypan blue exclusion method.

Soft agar colony formation assay. Cells (5×10^3) were seeded in 1 mL of 0.3% agar in complete medium in the presence of various doses of CsA over a 1-mL base layer of 0.6% agar in complete medium in six-well plates. Growth medium containing various doses of CsA was added and replaced every 2 or 3 d. Cells were incubated at 37°C for 3 to 4 wk. Colonies were photographed with a phase-contrast microscope. Colonies (≥50 μm) were counted with Image J software.

Cell motility and invasion assay. For cell invasion assay, cells were placed in a Matrigel-coated transwell chamber with 8-μm-pore positron emission tomography membranes in the presence of various doses of CsA and were allowed to migrate toward 3% fetal bovine serum for 24 h. The number of cells invading to the lower surface of the membrane was quantified by CyQuant (Invitrogen). Cell motility was assessed by two distinct assays. Transwell migration assays were carried out as described above for Matrigel invasion assays except that the chambers were not coated with Matrigel (26). For wound healing assay, the confluent cell monolayer was wounded with a p200 pipette tip and cultured in serum-free medium in the presence of various doses of CsA. Representative images of a wound closure assay were acquired with a phase-contrast microscope at the indicated times. The wound areas were measured using Image J and the percentage of the wound closed was calculated.

Xenograft model. MCF7 or MDA231 cells (1×10^6) were suspended in Matrigel and injected into the teat of the fourth abdominal mammary gland of nude (*nu/nu*) mice as described (28). In brief, following injection, animals were randomized and treated with control carrier (apple juice) or CsA (100 mg/kg/d, standard dose; doses from 5 to 300 mg/kg/d were also examined) 3 d postinjection for 12 wk by twice-daily gavage. The standard dose of CsA given the mice exceeded that received by human patients (human dose range, 0.2–10 mg/kg/d) given the rapidity at which CsA is excreted by the murine kidney. At no point in these experiments did mice receiving the standard dose (or less) show any signs of neurologic toxicity or weight loss. Tumor growth was measured by weekly caliper measurement. Mice xenografted with MCF7 cells received a subcutaneous estrogen pellet (Innovative Research) implant before injection to ensure the growth of this estrogen receptor (ER)–positive cell line. All animal experiments were conducted in accordance with a protocol approved by Northwestern University Institutional Animal Care and Use Committee. After 12 wk, the mice were euthanized by CO_2 followed by cervical dislocation, and the entire primary mammary tumors were removed and weighed. In addition, all visceral organs, brain, and superficial lymph nodes were harvested for microscopic examination for metastasis. One half of each tumor was immediately frozen in liquid nitrogen and then stored at −80°C. The remaining tumor and other organs (lung, lymph node, liver, etc.) removed from sacrificed animals were fixed in 10% formalin for subsequent H&E staining or immunohistochemistry, which was done by the Northwestern University Pathology Core Facility. The necrotic area of primary tumor was quantified by morphometric determination of the proportion of total tumor area that was necrotic in H&E-staining section as described above. Organ metastasis was detected by gross and histologic examination of H&E-staining section of organ tissues from the control mice and CsA-treated mice. The number of tumor nuclei labeling positive for cyclin D1 was quantified in four preassigned high-power fields per tumor by microscopic examination of anti–cyclin D1 immunohistochemistry of primary tumor tissues from mice treated with CsA or control vehicle.

Statistical analysis. Data were reported as the means ± SE and analyzed using the appropriated statistical methods as indicated. $P < 0.05$ was considered significant.

Results

PRL-induced gene expression is modulated by CypA levels and CsA treatment. To test whether CypA could serve as a molecular toggle for the PRLr, the effects of manipulating CypA levels and activity on both PRL-responsive gene reporters and

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endogenous genes were examined in breast cancer cells (Fig. 1). When overexpressed in T47D transfectants, CypA dose-dependently enhanced the PRL-induced expression from the Stat5-responsive LHRE and CISH luciferase reporters. These findings were paralleled by corresponding increases in the endogenous expression of CISH and cyclin D1 protein (Fig. 1*A* and *B*). Conversely, the suppression of endogenous CypA using a siRNA-mediated approach resulted in a decrease of PRL-induced gene expression as measured by LHRE and CISH luciferase reporters and endogenous CISH protein in T47D cells (Fig. 1*C* and *D*). Taken together, these findings show that alterations in CypA levels directly correlate with PRL-induced gene expression.

To examine whether the inhibitory effects of CypA knockdown were related to a reduction in the PPIase activity within the cell, T47D cells were treated with the PPI inhibitor CsA, followed by an analysis of PRL-induced signaling and gene expression evaluated (Fig. 2). As noted with the siRNA-mediated reduction of CypA, CsA treatment of T47D cells suppressed both basal and PRL-induced expression of endogenous CISH and cyclin D1 (Fig. 2*B*). To further delineate a mechanism for the reduction in PRL-induced gene expression mediated by CsA treatment, analysis of PRL-induced signaling was done. These studies (Fig. 2*A*) revealed a marked inhibition of PRL-stimulated phosphorylation of Jak2, Stat5, Erk1/2, and Akt. PRL-induced activation of these signaling molecules was also significantly inhibited at lower doses (i.e., 1 μg/mL; data not shown). Because many of the functions of the PRLr require and are initially triggered by Jak2 (29, 30), these findings would suggest that PPI activity contributes to proximal PRLr signaling.

To confirm this hypothesis, several follow-up and control experiments were done. If CsA was inhibiting the action of CypA at the PRLr, then one could anticipate that CsA treatment could interfere with CypA-PRLr interaction. Indeed, this effect was noted (Fig. 2*C*), as minimal CypA could be detected in the anti-PRLr or anti-Jak2 immunoprecipitates from CsA-treated T47D cells. In contrast, CsA had no effect on the PRLr-Jak2 interaction (Supplementary Fig. S1). The effects of CsA on receptor-mediated signaling were specific, as parallel experiments examining the activation of Erk1/2 and Akt CsA-treated T47D cells showed no significant diminution of insulin- or epidermal growth factor–triggered signaling (Supplementary Fig. S2). In addition, CsA had no effect on PRL binding to the PRLr (Supplementary Fig. S3). Thus, taken as a whole, these data support the notion that PPI activity, such as that found in CypA, significantly contributes to PRLr-induced signal transduction and gene expression. Interestingly, CsA also inhibits growth hormone–induced Akt and Erk signaling (Supplementary Fig. S2). Given that the growth hormone and PRL receptors are highly related members of the cytokine receptor superfamily that use similar signaling pathways, this observation is not surprising and is an area of ongoing research.

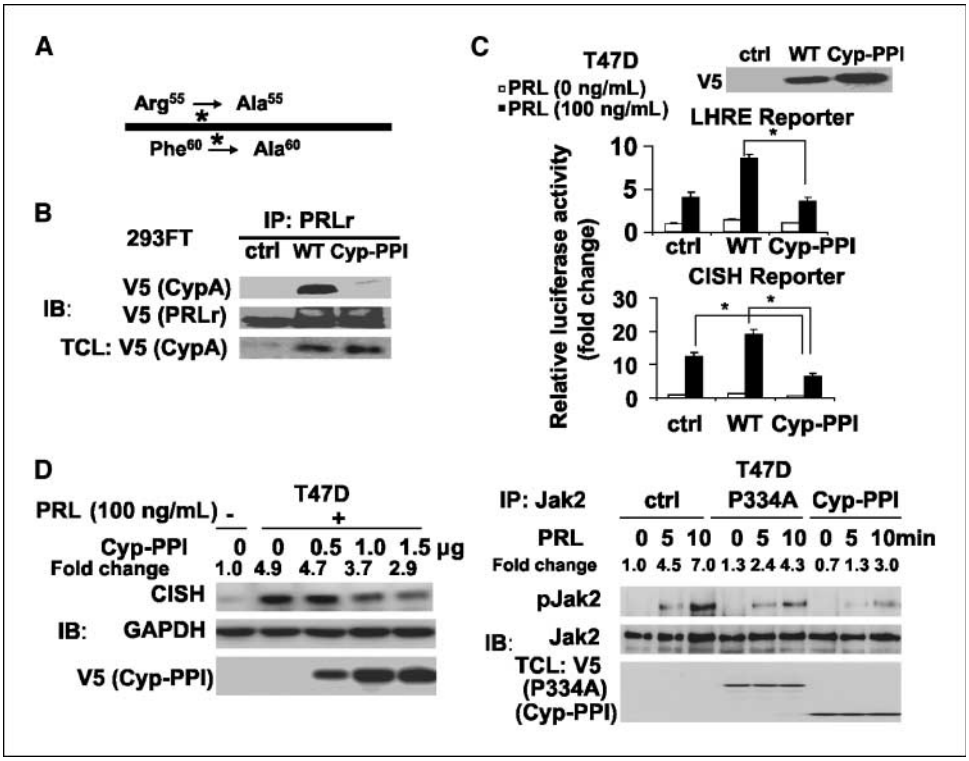

Figure 3. Overexpression of PPI-defective mutant of CypA is unable to associate with the PRLr and potentiate PRL-induced gene expression. *A,* generation of a PPI-defective CypA mutant (Cyp-PPI). Two residues (Arg^{55} and Phe^{60}) were mutated to Ala using site-directed mutagenesis. *B,* Cyp-PPI is unable to bind to the PRLr. Wild-type (*WT*) CypA, its mutant (*Cyp-PPI*), or empty vector control (*ctrl*) was coexpressed with the PRLr in 293FT transfectants and immunoprecipitated by using an antibody against PRLr-ECD. Cell lysates and immunoprecipitates were probed in immunoblots with the indicated antibodies. *C,* Cyp-PPI fails to potentiate PRL-induced gene expression. T47D cells were cotransfected with LHRE or CISH luciferase reporter and wild-type CypA, Cyp-PPI, or empty vector expression construct. Following 24-h stimulation with PRL (100 ng/mL), transfectants were lysed and assayed for luciferase activity. *Columns,* mean of two independent experiments; *bars,* SE. *, $P < 0.05$ (*t* test). Western blot analysis (*top*) of total cell lysates with an anti-V5 antibody was done to verify the expression of transfected CypA. *D,* Cyp-PPI inhibits PRL-induced endogenous CISH protein expression (*left*), and Cyp-PPI and PRLr P334A inhibit Jak2 phosphorylation (*right*). *Left,* suppression of endogenous, PRL-induced CISH protein expression by the overexpression of Cyp-PPI was detected by Western blot analysis. The cells were treated with PRL (100 ng/mL) for 24 h. *Right,* immunoprecipitation and immunoblots of T47D cells overexpressing either Cyp-PPI or PRLr P334A (or empty vector control) were done as indicated.

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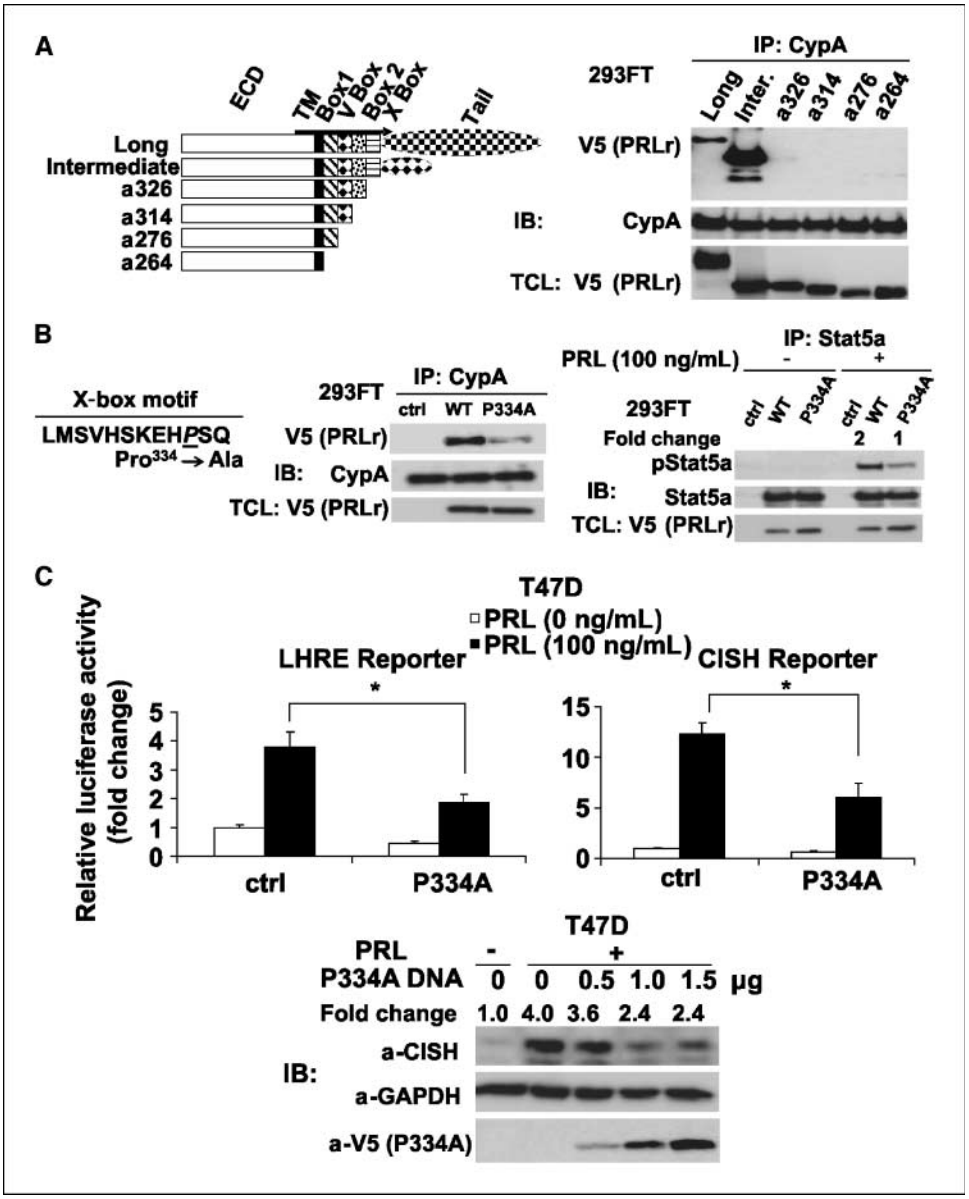

Figure 4. Mapping of the CypA-binding site on the PRLr results in the generation of an interaction-defective point mutant PRLr that functions as a dominant negative receptor. *A,* CypA binding requires the PRLr X-box. A set of the PRLr COOH-terminal truncation mutants were prepared using PCR-based mutagenesis. 293FT transfectant lysates were immunoprecipitated with an anti-CypA antibody and sequentially immunoblotted with an anti-V5 antibody. *B, left,* analysis of the PRLr X-Box reveals a conserved proline residue. PCR mutagenesis was used to generate a receptor termed PRLr P334A. *Middle,* the association of CypA with the PRLr P334A is markedly decreased. 293 transfectants overexpressing wild-type PRLr and the PRLr P334A mutants were immunoprecipitated with an anti-CypA antibody and analyzed by immunoblot analyses as indicated. *Right,* Stat5 activation by PRLr P334A is significantly impaired. Wild-type PRLr and its PRLr P334A were coexpressed with rabbit Stat5a in 293FT transfectants. The cells were stimulated with PRL (100 ng/mL) for 10 min and immunoprecipitated with an antibody against Stat5. Total cell lysates and immunoprecipitates were probed with the indicated antibodies and subjected to Western blot analyses as indicated. *C,* PRLr P334A inhibits PRL-induced gene expression in T47D cells in a dominant negative manner. *Top,* T47D cells were transfected with LHRE or CISH luciferase reporters and a PRLr P334A expression construct (or empty vector control). Following 24-h stimulation with PRL (100 ng/mL), transfectants were lysed and assayed for luciferase activity. *Columns,* mean of two independent experiments; *bars,* SE. *, $P < 0.05$ (*t* test). *Bottom,* PRLr P334A inhibits PRL-induced endogenous CISH protein expression. Suppression of endogenous, PRL-induced CISH protein levels by PRLr P334A transfectants was detected by Western blot analysis.

The PPIase activity of CypA is required for potentiation of PRL-induced gene expression and association of CypA with the PRLr. To delineate the molecular basis for interaction between the PRLr and CypA and correlate this to function, selective mutagenesis was done. Many of the interactions of cyclophilins are mediated by the PPI active site (31), and the inhibition of PRLr-CypA binding by CsA further suggested that the PPI pocket was involved in this event. Point mutagenesis to residues Arg^{55} and Phe^{60} of the PPI pocket of CypA (Fig. 3*A*) resulted in a mutant CypA lacking 99% of PPI activity found in wild-type CypA (32). Subsequent analysis then queried whether this PPI-defective form of CypA was capable of interacting with the PRLr and potentiating its signal, as observed with wild-type CypA. When coexpressed with the PRLr in 293FT cells, unlike wild-type CypA, Cyp-PPI interacted poorly with the PRLr (Fig. 3*B*). Furthermore, in contrast to wild-type CypA, which potentiated both the PRL-induced LHRE and CISH luciferase reporters when overexpressed in T47D cells, overexpressed Cyp-PPI failed to potentiate PRL-induced gene expression of the LHRE (Fig. 3*C*). Indeed, Cyp-PPI overexpression significantly repressed gene expression from CISH luciferase reporter (Fig. 3*C*) and blocked Jak2 phosphorylation and the endogenous expression of CISH protein (Fig. 3*D*). These data show that the PPIase active site of CypA is involved in the PRLr-CypA interaction and that this PPIase activity is required for the potentiation of PRL-induced gene expression.

A conserved proline residue in the PRLr X-box motif contributes to CypA binding and is required for PRLr transduction. The studies presented above reveal a proximal contribution of the PPI activity of CypA to PRLr/Jak2 signaling. However, the possibility remained that the action of CypA could be indirect, acting at a site other than the PRLr to effect its potentiation of Jak2/Stat5 signaling. To address this question, the binding site of CypA on the PRLr was mapped. As an initial step, COOH-terminal PRLr truncation mutants were generated and tested for their ability when expressed to interact with CypA (Fig. 4*A*). These studies revealed that truncations of the PRLr membrane proximal

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to the X-box motif were incapable of interacting with CypA, revealing a contribution of the X-box to the engagement of CypA. Analysis of the X-box motif (Fig. 4*B*) revealed a proline at residue 334 of the PRLr that was highly conserved across species. Given the affinity of cyclophilins for proline residues, it was hypothesized that P334 in the X-box could contribute to the engagement of CypA. To test this hypothesis, a proline-to-alanine replacement mutation of the PRLr was generated (P334A), and the ability of this mutant PRLr to interact with CypA and mediate PRL-induced signaling was tested. These studies revealed that when expressed in T47D cells, the mutant PRLr P334A showed a significantly reduced association with CypA (Fig. 4*B*). Furthermore, the ability of the P334A mutant to induce Jak2 and Stat5 phosphorylation (in comparison with wild-type PRLr transfected control) was also significantly diminished (Figs. 3*D* and 4*B*). Overexpression of the PRLr P334A mutant in T47D cells resulted in a dose-dependent reduction in the PRL-induced expression of both cotransfected LHRE and CISH luciferase reporters and endogenous CISH protein

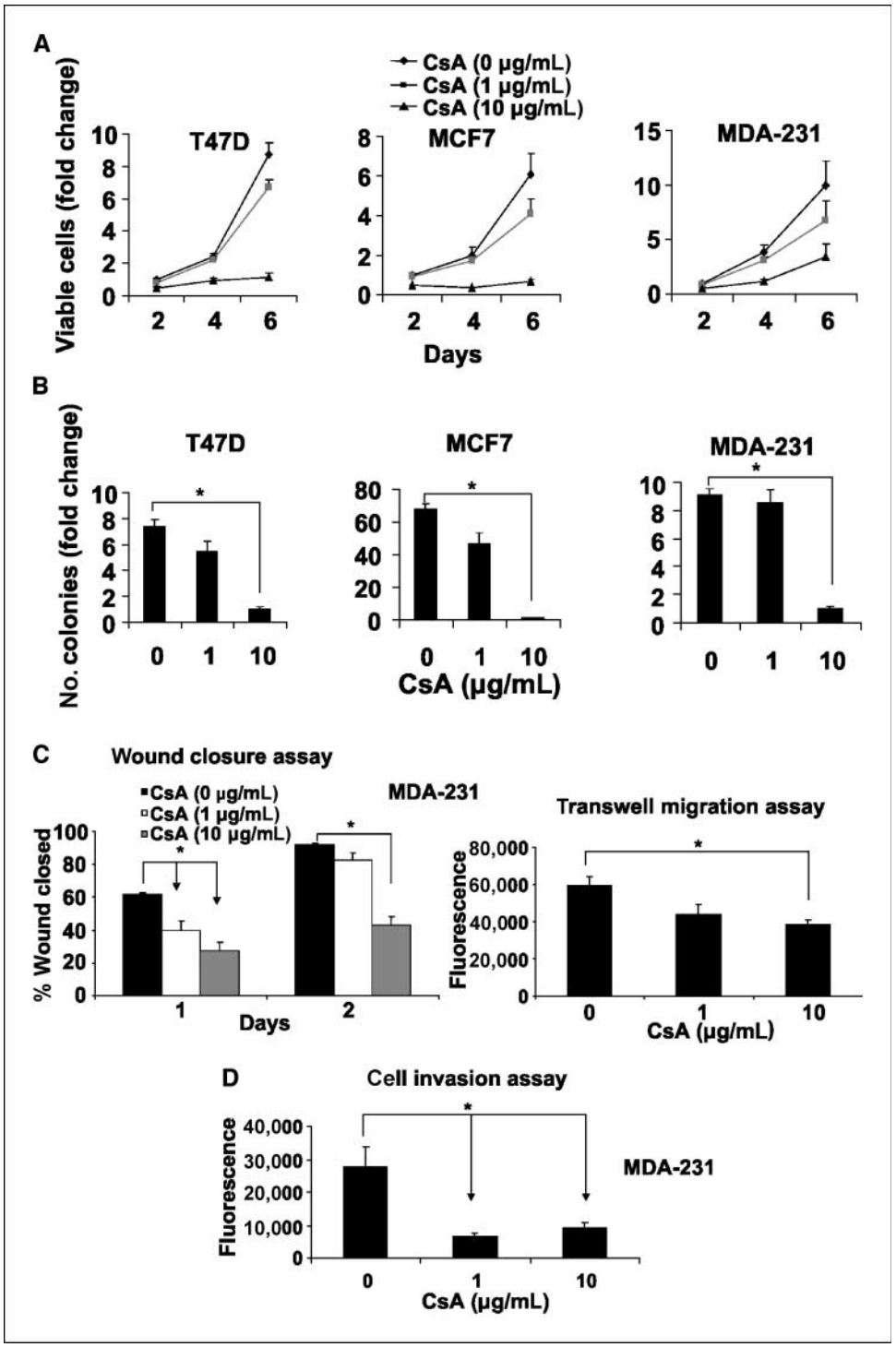

Figure 5. CsA inhibits anchorage-dependent and anchorage-independent growth and motility and invasion of breast cancer cells *in vitro*. *A,* growth inhibition by CsA of breast cancer cells. T47D, MCF7, and MDA-231 cells were incubated with various doses of CsA, and viable cells were quantified every other day by trypan blue exclusion. *Points,* mean of two independent experiments; *bars,* SE. *B,* colony formation inhibition by CsA. Colony enumeration used Image J software. *Columns,* mean of two independent experiments; *bars,* SE. *, $P < 0.05$ (*t* test). *C,* inhibition of motility by CsA as measured by wound healing assay (*left*) and Boyden chamber assay (*right*). *Left,* a confluent cell monolayer of MDA-231 was wounded with a pipette tip and cultured in serum-free medium in the presence of various doses of CsA. Representative images of a wound closure assay were acquired with a phase-contrast microscope and the percentage of the wound closed was quantified. Representative of three independent experiments. *, $P < 0.05$ (two-way ANOVA). *Right,* the number of cells migrating through pores was quantified by CyQuant labeling. Representative of three independent experiments. *, $P < 0.05$ (one-way ANOVA). *D,* inhibition of invasion by CsA. The number of MDA-231 cells invading through Matrigel-occluded pores was quantified by CyQuant labeling. Representative of three independent experiments. *, $P < 0.05$ (one-way ANOVA).

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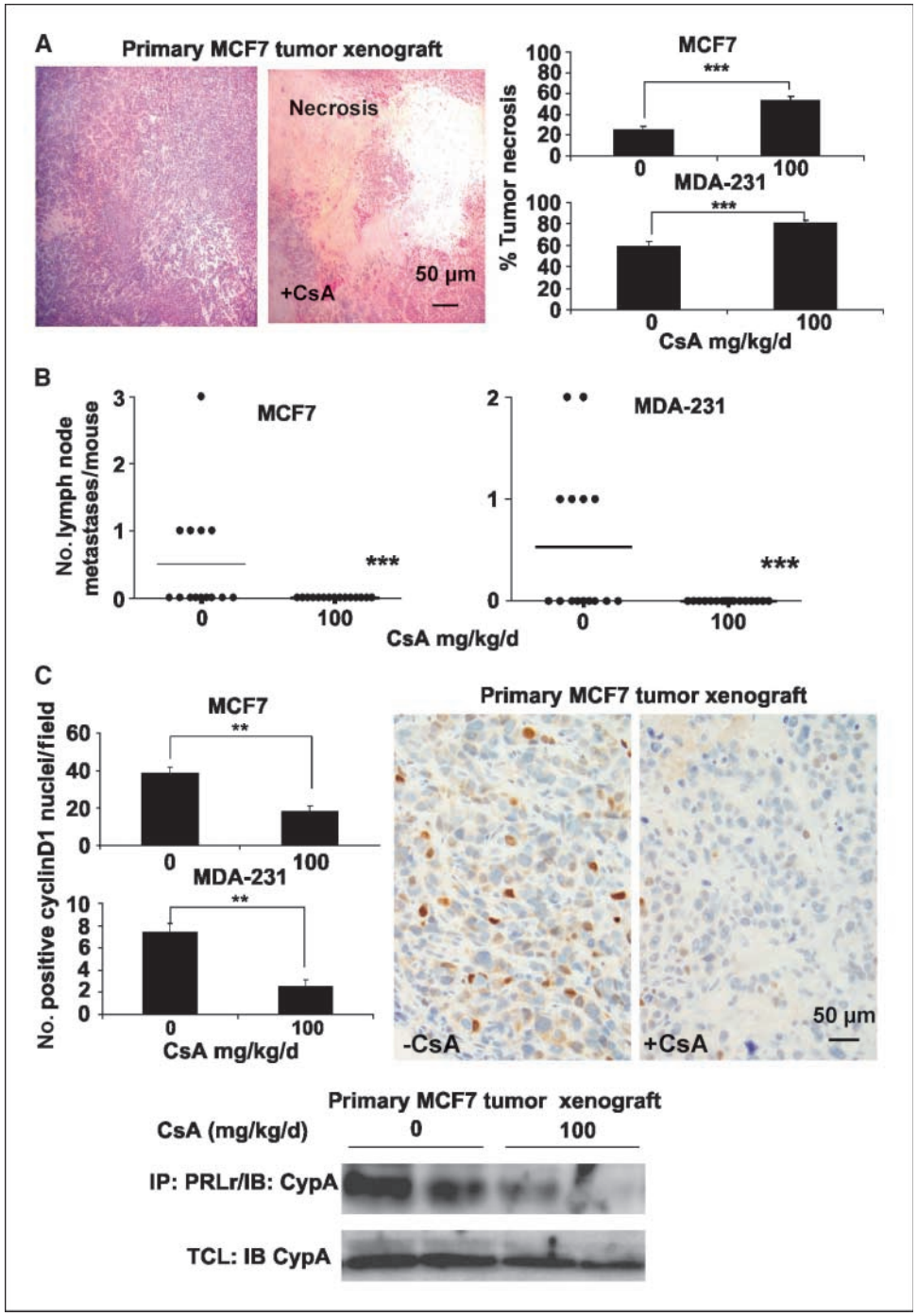

Figure 6. CsA induces central primary tumor necrosis and the metastasis of ER^+ and ER^- human breast cancer xenografts, inhibits primary tumor cyclin D1 expression, and blocks CypA-PRLr association. *A,* induction of primary tumor necrosis by CsA. Mice xenografted with MCF7 and MDA231 cells were treated with CsA (100 mg/kg/d; $n = 18$) or control carrier (apple juice; $n = 18$) for 12 wk by twice-daily gavage. *Left,* representative H&E-stained sections (20×; *bar,* 50 μm). *Right,* the necrotic area of primary tumor (*right*) was quantified by determination of the proportion of total tumor area that was necrotic in H&E-stained section of primary MCF7 tumor xenograft. ***, $P < 0.001$ (*t* test). *B,* inhibition of metastasis by CsA. Lymph node metastases were detected by histologic examination of H&E-stained section of lymph nodes harvested from mice treated for 12 wk with either CsA (100 mg/kg/d; $n = 18$) or control carrier (apple juice; $n = 18$). ***, $P < 0.001$ (Fisher's exact test). *C,* inhibition of primary tumor cyclin D1 expression (*top*) and CypA-PRLr association (*bottom*) by CsA. *Top,* the number of positive cyclin D1 nuclei was quantified by microscopic examination of anti–cyclin D1 immunohistochemical labeling of primary tumor tissue from mice treated for 12 wk with either CsA (100 mg/kg/d; $n = 5$) or control carrier (apple juice; $n = 5$). **, $P < 0.01$ (*t* test). Representative sections (*bar,* 50 μm) of anti–cyclin D1 immunohistochemical labeling of primary MCF7 tumor xenograft. *Columns,* mean; *bars,* SE. *Bottom,* CsA blocks the association of CypA with the PRLr in primary MCF7 tumor xenograft. The inhibition of CypA-PRLr association was detected by coimmunoprecipitation analysis.

(Fig. 4*C*). These findings are notable in that T47D cells are known to express high levels of endogenous PRLr, indicating that the P334A mutant, most probably through heterodimerization with the wild-type PRLr (33), functions as a dominant negative receptor. When assessed with the data presented above, these findings would indicate that the direct action of the CypA on the PRLr is required for effective PRL-induced signaling, a process that is inhibited by the PRLr P334A mutant. These findings were further confirmed by control experiments, which showed that the PRLr/Jak2 interaction was not affected by the P334A mutation (data not shown).

CsA treatment blocks multiple cellular functions in breast cancer cells *in vitro*. The data presented above have revealed that the function of the PRLr and its associated downstream signaling are regulated by the PPIase activity of CypA. Thus, given the function of cyclophilins, not only at the level of the PRLr but at other signaling loci that contribute to the pathogenesis of breast cancer (i.e., such as NFAT ref. 34 and Stat 5 ref. 12, 25) as well, it was reasoned that the use of PPI inhibitors such as CsA might effectively inhibit the malignant phenotype of breast cancer. To assess the *in vitro* effects of CsA on the biology of human breast cancer, analysis of the effects of CsA on the cell

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growth, soft agar colony formation, cell motility, and invasion was done. When cultured in monolayer in the presence of CsA, both the ER^+ lines T47D and MCF7 and the ER^- line MDA-231 showed a dose-dependent inhibition of viable cell growth (Fig. 5*A*). At concentrations ≤10 μg/mL, this antiproliferative effect was reversible following washing and medium change; at concentrations of ≥30 μg/mL, these effects were not reversible and cell death ensued (data not shown). To assess the effects of CsA on anchorage-independent growth, soft agar colony formation assays were done. As shown in Fig. 5*B*, CsA treatment resulted in a marked suppression of the anchorage-independent growth of human breast cancer cells in a dose-dependent manner. When introduced into either cell migration or invasion assays using the highly metastatic ER^- MDA-231, CsA dose-dependently inhibited both cell motility [as assessed by wound healing assay (Fig. 5*C*, *left*; Supplementary Fig. S4) or Boyden chamber assay (Fig. 5*C*, *right*)] and cell invasion through Matrigel (Fig. 5*D*). Taken together, these findings indicate that CsA inhibited many of the malignant properties of breast cancer cells *in vitro*, suggesting a potential *in vivo* role for PPI inhibition in the treatment of breast cancer.

Inhibition of PPI activity *in vivo* by CsA induces primary tumor necrosis and blocks metastasis of breast cancer xenografts. Given the *in vitro* effects of CsA on breast cancer cell biology, it was hypothesized that CsA could inhibit the *in vivo* outgrowth of human breast cancer. To test this hypothesis, both the ER^+ MCF7 and the ER^- MDA-231 human breast cancer lines were xenografted into nude mice, using a novel approach that dramatically facilitates the metastatic spread of breast cancer xenografts (28), and subsequently randomized into CsA therapy or control groups 3 days postinjection. Whereas all CsA-treated tumors showed a trend toward a decrease in size and weight over time, these parameters did not achieve statistical significance (data not shown). However, two parameters were found to be significantly changed as a function of CsA therapy, notably central tumor necrosis and metastasis. As seen in Fig. 6*A*, central tumor necrosis of both ER^+ and ER^- was significantly increased, with a somewhat more pronounced effect in ER^+ MCF7 tumors. Most interestingly, not a single metastasis to lymph nodes or organs was noted in CsA-treated mice (Fig. 6*B*) from either ER^+ or ER^- xenografts. Indeed, in this entire line of experimentation with 94 mice receiving varied doses of CsA (some as low as 10 mg/kg/d), not a single CsA-treated mouse xenografted with either MCF7 or MDA-231 cells was noted to have a metastasis, whereas between 30% and 50% of the control mice showed metastasis. To address whether the *in vivo* use of CsA resulted in inhibition of Stat5-driven gene expression, immunohistochemistry of the primary tumors with an anti–cyclin D1 antibody was done and the number of labeled nuclei was quantified. As shown in Fig. 6*C* (*top*), *cyclin D1* gene expression in both ER^+ and ER^- primary tumors from CsA-treated mice was significantly inhibited when compared with tumors from control mice. To further support the *in vitro* observation that the association of CypA with the PRLr was blocked by CsA, the *in vivo* status of PRLr-CypA binding in CsA-treated tumor tissues was also examined. As shown in Fig. 6*C* (*bottom*), the CypA-PRLr association in CsA-treated tumor tissues compared with control vehicle–treated tumor tissues was inhibited. Thus, the *in vivo* actions of CsA, while reducing cyclin D1 expression and blocking CypA-PRLr association, also promoted central tumor necrosis and inhibited metastasis of breast cancer xenografts, irrespective of ER status.

Discussion

The proximate mechanisms that enable receptor-mediated signal transduction following ligand binding remain poorly characterized. Following ligand binding to the type I cytokine receptors, such as the PRLr and growth hormone receptor, members of the Jak family of tyrosine kinases are activated within 30 to 60 seconds by an autophosphorylation-based mechanism (35). Recent analysis of the growth hormone receptor using mutagenesis and fluorescence resonance energy transfer approaches has suggested that a conformational change may occur within the receptor following ligand binding approximating the growth hormone receptor–associated Jak2 kinases, thereby enabling their autophosphorylation and activation (20). Nuclear magnetic resonance spectroscopy of the conserved Box 1 motif of the PRLr, a hydrophobic and proline-rich intracellular domain adjacent to the transmembrane region of this receptor, also has suggested that a conformational change within the PRLr is also feasible (21). However, a mechanism that would enable such a conformational change to occur within either the growth hormone receptor or PRLr has not been elaborated.

The data presented here indicate that the PPI activity of CypA significantly contributes to proximate receptor activation, enabling transduction through the PRLr/Jak2 complex. The PRL-induced expression of both Stat5-responsive reporter constructs and endogenous genes was directly correlated to CypA levels by both overexpression and knockdown approaches. Loss of PPI activity in CypA (CypA-PPI) following mutagenesis resulted in reduced PRLr binding, Jak2 phosphorylation, and PRL-induced gene expression. Similarly, replacement by mutation of a conserved proline residue (PRLr-P334A) also resulted in reduced PRLr-CypA interaction, decreased Jak2 and Stat5 phosphorylation, and decreased Stat5-responsive reporter and endogenous gene expression. Indeed, when transfected into the T47D breast cancer line, which expresses high levels of PRLr, the PRLr-P334A functioned in a dominant negative manner. Taken together, these results would argue that the potentiation of PRLr-induced signaling by CypA is a consequence of the direct actions of the PPI of CypA on the PRLr/Jak2 complex, and not an epiphenomenon of CypA function at another site.

As a member of the cytokine receptor superfamily, the membrane-proximal portion of the intracellular domain of the PRLr contains the conserved Box 1/Variable Box/Box 2/X-box motifs. Whereas the functions of the Variable Box, Box 2, and X-Box are largely uncharacterized, the proline-rich Box 1 motif has been implicated in the binding and ligand-induced activation of Jak2. Deletion of this motif from the PRLr or nonconservative replacement of its COOH-terminal proline residue with leucine results in a loss of Jak2 binding via its NH_2-terminal FERM domain (36) and downstream PRL-induced gene expression (37). However, although necessary for Jak2 engagement, the Box 1 motif alone is not sufficient for lactogenic signaling (33, 37), clearly indicating that other determinants in the PRLr contribute to Jak2 activation. The identified contribution of the proline residue at position 334 for both CypA-PRLr association and PRL-induced Jak2 activation would indicate that the PPI activity of CypA at this site or its immediate environs (such as the Box 1 motif) immediately affects Jak2-driven signaling.

CsA has been a widely used immunosuppressive drug in organ transplantation and in the treatment of autoimmune diseases (38, 39). As an immunosuppressant, CsA has been classically thought to form a complex with cyclophilin that binds with high

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affinity to the phosphatase calcineurin. As a consequence of this event, interleukin-2 transcription is inhibited by a loss of calcineurin-mediated dephosphorylation of the transcription factor NFAT activation, resulting in a loss of clonal T-cell proliferation following antigen stimulation (40). However, our data would indicate that, at least in human breast cancer cells, CsA also targets the proximal Jak2 kinase, presumably through its inhibition of the PPI activity of CypA. The loss of Jak2 activity readily explains the loss of PRL-induced activation of downstream signaling factors such as Stat5, Akt, and Erk, and the concomitant loss of PRL-induced, Stat5-mediated gene expression and the inhibition of outgrowth of CsA-treated breast cancer cells. Was the inhibition of breast cancer cell outgrowth secondary to the effects of CsA on Stat5 or NFAT? *In vitro* evidence does exist showing that NFAT activity regulated by the Akt kinase stimulates breast cancer motility and invasion (34, 41). However, the precise role of this transcription factor in either normal breast biology or breast cancer pathobiology *in vivo* remains uncertain. In contrast, *in vivo* studies using conditional Jak2$^{-/-}$ knockout mice, and cells derived thereof, have revealed that Jak2 is required for normal mammary alveogenesis and lactation at the tissue level and the activation of Stat5 and expression of cyclin D1 at the cellular level (29, 30). Furthermore, when transgenic WAP-T antigen mice, a genetic model of mammary cancer, were mated to heterozygous Stat5$^{+/-}$ mice, a significant reduction in mammary tumor size, incidence, and progression was noted (42). Most importantly, our knockdown and overexpression studies presented here (using both wild-type and mutant CypA and PRLr) showed the effects on signaling and gene expression comparable to those observed following CsA treatment. These complementary, non–CsA-based approaches, which should not result in the chelation of calcineurin or the inhibition of other cyclophilin family members, would argue the importance of the Jak2/Stat5 axis in breast cancer pathobiology and suggest that the inhibition of this pathway by CsA may, in part, explain the actions of this drug both *in vitro* and *in vivo*. However, it is important to note that the inhibitory effects of CsA may be unique to breast malignancies, as CsA is recognized to increase or enhance the incidence and progression of epidermal, lymphoid, and gastrointestinal malignancies in the laboratory and the clinic (43–45).

Our *in vitro* studies showed that CsA markedly inhibited the growth, motility, and invasion of both ER$^+$ and ER$^-$ human breast cancer cells in a dose-dependent manner. Interestingly, *in vivo* CsA therapy did not have a statistically significant effect on overall tumor size (although CsA-treated tumors did trend toward being smaller). However, a statistically significant increase in central primary tumor necrosis and a complete absence of metastasis were noted in CsA-treated mice xenografted with either ER$^+$ or ER$^-$ breast cancer cells. Significantly, as noted *in vitro*, CsA-treated xenografts showed a significant reduction in the expression of the Stat5-responsive cyclin D1. There are many potential mechanisms through which these CsA-mediated effects on xenograft progression may have been mediated. In this context, it is interesting to note that the inhibition of cyclin D1 expression is angiostatic (46). In addition, epidemiologic studies have implicated a role for PRL in the metastatic progression of human breast cancer (47, 48). It is tempting to speculate, therefore, that some of the progression-inhibitory effects of CsA may result from its blockade of the PRL/Jak2/Stat5 pathway, the activity of which has been implicated in the proliferation, survival, motility, and invasion of human breast cancer (17). It is also important to note that the significant reduction in breast cancer observed in female patients undergoing CsA therapy following allograft transplant (16) was at that time interpreted to be secondary to the immunosuppressive effects of CsA. Our *in vitro* and *in vivo* data in models lacking functional immune systems, however, would argue that some, if not many, of the effects of CsA are due to its direct action on breast cancer cells, and not due to its secondary immunosuppressive actions.

In many tumor types, including breast cancer, CsA has been found to bind to and inhibit the efflux functions of multidrug resistance proteins including P-glycoprotein, breast cancer resistance protein, and multidrug resistance protein (49, 50). First documented in Chinese hamster ovary cells (51) and extended into human leukemias (52), these studies have served as the basis for the first phase II clinical trials with cyclosporine in patients with advanced breast cancer. In theory, by reducing drug efflux, thereby enhancing the efficacy of chemotherapy, the combination of Paxil and CsA was found to be safe and effective in patients with advanced disease (53) when administered simultaneously on a once-weekly basis. Our findings, however, would argue that although CsA may also function as a drug efflux inhibitor, this PPI inhibitor has direct actions on breast cancer signaling that should be exploited in patients with this disease, by establishing continuous serum levels of CsA that would inhibit Jak2/Stat5 transduction. Indeed, as alternative PPI-inhibitors continue to be developed, novel and more specific pharmacotherapies against breast cancer should be expected.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Gothel SF, Marahiel MA. Peptidyl-prolyl *cis-trans* isomerases, a superfamily of ubiquitous folding catalysts. Cell Mol Life Sci 1999;55:423–36.
2. Handschumacher RE, Harding MW, Rice J, Drugge RJ, Speicher DW. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science 1984;226:544–7.
3. Bram RJ, Hung DT, Martin PK, Schreiber SL, Crabtree GR. Identification of the immunophilins capable of mediating inhibition of signal transduction by cyclosporin A and FK506: roles of calcineurin binding and cellular location. Mol Cell Biol 1993;13:4760–9.
4. Colgan J, Asmal M, Yu B, Luban J. Cyclophilin A-deficient mice are resistant to immunosuppression by cyclosporine. J Immunol 2005;174:6030–8.
5. Harrison RK, Stein RL. Substrate specificities of the peptidyl prolyl *cis-trans* isomerase activities of cyclophilin and FK-506 binding protein: evidence for the existence of a family of distinct enzymes. Biochemistry 1990;29:3813–6.
6. Lu KP, Finn G, Lee TH, Nicholson LK. Prolyl *cis-trans* isomerization as a molecular timer. Nat Chem Biol 2007;3:619–29.
7. Bosco DA, Eisenmesser EZ, Pochapsky S, Sundquist WI, Kern D. Catalysis of *cis/trans* isomerization in native HIV-1 capsid by human cyclophilin A. Proc Natl Acad Sci U S A 2002;99:5247–52.
8. Colgan J, Asmal M, Neagu M, et al. Cyclophilin A regulates TCR signal strength in CD4$^+$ T cells via a proline-directed conformational switch in Itk. Immunity 2004;21:189–201.

DOI:10.1158/0008-5472.CAN-08-0639

9. Nicholson LK, Lu KP. Prolyl *cis-trans* isomerization as a molecular timer in Crk signaling. Mol Cell 2007;25:483–5.
10. Lummis SC, Beene DL, Lee LW, Lester HA, Broadhurst RW, Dougherty DA. *Cis-trans* isomerization at a proline opens the pore of a neurotransmitter-gated ion channel. Nature 2005;438:248–52.
11. Eckert B, Martin A, Balbach J, Schmid FX. Prolyl isomerization as a molecular timer in phage infection. Nat Struct Mol Biol 2005;12:619–23.
12. Rycyzyn MA, Clevenger CV. The intranuclear prolactin/cyclophilin B complex as a transcriptional inducer. Proc Natl Acad Sci U S A 2002;99:6790–5.
13. Campa MJ, Wang MZ, Howard B, Fitzgerald MC, Patz EF, Jr. Protein expression profiling identifies macrophage migration inhibitory factor and cyclophilin a as potential molecular targets in non-small cell lung cancer. Cancer Res 2003;63:1652–6.
14. Shen J, Person MD, Zhu J, Abbruzzese JL, Li D. Protein expression profiles in pancreatic adenocarcinoma compared with normal pancreatic tissue and tissue affected by pancreatitis as detected by two-dimensional gel electrophoresis and mass spectrometry. Cancer Res 2004;64:9018–26.
15. Rey O, Baluda MA, Park NH. Differential gene expression in neoplastic and human papillomavirus-immortalized oral keratinocytes. Oncogene 1999;18:827–31.
16. Stewart T, Tsai SC, Grayson H, Henderson R, Opelz G. Incidence of de-novo breast cancer in women chronically immunosuppressed after organ transplantation. Lancet 1995;346:796–8.
17. Clevenger CV, Furth PA, Hankinson SE, Schuler LA. The role of prolactin in mammary carcinoma. Endocr Rev 2003;24:1–27.
18. Hankinson SE, Willett WC, Michaud DS, et al. Plasma prolactin levels and subsequent risk of breast cancer in postmenopausal women. J Natl Cancer Inst 1999;91:629–34.
19. Rose-Hellekant TA, Arendt LM, Schroeder MD, Gilchrist K, Sandgren EP, Schuler LA. Prolactin induces ERα-positive and ERα-negative mammary cancer in transgenic mice. Oncogene 2003;22:4664–74.
20. Brown RJ, Adams JJ, Pelekanos RA, et al. Model for growth hormone receptor activation based on subunit rotation within a receptor dimer. Nat Struct Mol Biol 2005;12:814–21.
21. O'Neal KD, Chari MV, McDonald CH, et al. Multiple *cis-trans* conformers of the prolactin receptor proline-rich motif (PRM) peptide detected by reverse-phase HPLC, CD and NMR spectroscopy. Biochem J 1996;315:833–44.
22. Waters MJ, Hoang HN, Fairlie DP, Pelekanos RA, Brown RJ. New insights into growth hormone action. J Mol Endocrinol 2006;36:1–7.
23. Brockman JL, Schroeder MD, Schuler LA. PRL activates the cyclin D1 promoter via the Jak2/Stat pathway. Mol Endocrinol 2002;16:774–84.
24. Raccurt M, Tam SP, Lau P, et al. Suppressor of cytokine signalling gene expression is elevated in breast carcinoma. Br J Cancer 2003;89:524–32.
25. Rycyzyn MA, Reilly SC, O'Malley K, Clevenger CV. Role of cyclophilin B in prolactin signal transduction and nuclear retrotranslocation. Mol Endocrinol 2000;14:1175–86.
26. Miller SL, DeMaria JE, Freier DO, Riegel AM, Clevenger CV. Novel association of Vav2 and Nek3 modulates signaling through the human prolactin receptor. Mol Endocrinol 2005;19:939–49.
27. Syed F, Rycyzyn MA, Westgate L, Clevenger CV. A novel and functional interaction between cyclophilin A and prolactin receptor. Endocrine 2003;20:83–90.
28. Harrell JC, Dye WW, Allred DC, et al. Estrogen receptor positive breast cancer metastasis: altered hormonal sensitivity and tumor aggressiveness in lymphatic vessels and lymph nodes. Cancer Res 2006;66:9308–15.
29. Sakamoto K, Creamer BA, Triplett AA, Wagner KU. The Janus kinase 2 is required for expression and nuclear accumulation of cyclin D1 in proliferating mammary epithelial cells. Mol Endocrinol 2007;21:1877–92.
30. Wagner KU, Krempler A, Triplett AA, et al. Impaired alveologenesis and maintenance of secretory mammary epithelial cells in Jak2 conditional knockout mice. Mol Cell Biol 2004;24:5510–20.
31. Ke HM, Zydowsky LD, Liu J, Walsh CT. Crystal structure of recombinant human T-cell cyclophilin A at 2.5 A resolution. Proc Natl Acad Sci U S A 1991;88:9483–7.
32. Zydowsky LD, Etzkorn FA, Chang HY, et al. Active site mutants of human cyclophilin A separate peptidyl-prolyl isomerase activity from cyclosporin A binding and calcineurin inhibition. Protein Sci 1992;1:1092–9.
33. Chang WP, Clevenger CV. Modulation of growth factor receptor function by isoform heterodimerization. Proc Natl Acad Sci U S A 1996;93:5947–52.
34. Jauliac S, Lopez-Rodriguez C, Shaw LM, Brown LF, Rao A, Toker A. The role of NFAT transcription factors in integrin-mediated carcinoma invasion. Nat Cell Biol 2002;4:540–4.
35. Brooks AJ, Wooh JW, Tunny KA, Waters MJ. Growth hormone receptor; mechanism of action. Int J Biochem Cell Biol. In press 2008.
36. He K, Wang X, Jiang J, et al. Janus kinase 2 determinants for growth hormone receptor association, surface assembly, and signaling. Mol Endocrinol 2003;17:2211–27.
37. Lebrun JJ, Ali S, Ullrich A, Kelly PA. Proline-rich sequence-mediated Jak2 association to the prolactin receptor is required but not sufficient for signal transduction. J Biol Chem 1995;270:10664–70.
38. Kahan BD. Cyclosporine. N Engl J Med 1989;321:1725–38.
39. Ponticelli C. Cyclosporine: from renal transplantation to autoimmune diseases. Ann N Y Acad Sci 2005;1051:551–8.
40. Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK506. Immunol Today 1992;13:136–42.
41. Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A. Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. Mol Cell 2005;20:539–50.
42. Ren S, Cai HR, Li M, Furth PA. Loss of Stat5a delays mammary cancer progression in a mouse model. Oncogene 2002;21:4335–9.
43. Hojo M, Morimoto T, Maluccio M, et al. Cyclosporine induces cancer progression by a cell-autonomous mechanism. Nature 1999;397:530–4.
44. Ohsawa I, Murakami T, Uemoto S, Kobayashi E. *In vivo* luminescent imaging of cyclosporin A-mediated cancer progression in rats. Transplantation 2006;81:1558–67.
45. Koehl GE, Andrassy J, Guba M, et al. Rapamycin protects allografts from rejection while simultaneously attacking tumors in immunosuppressed mice. Transplantation 2004;77:1319–26.
46. Hanai J, Dhanabal M, Karumanchi SA, et al. Endostatin causes G_1 arrest of endothelial cells through inhibition of cyclin D1. J Biol Chem 2002;277:16464–9.
47. Wang DY, Stepniewska KA, Allen DS, et al. Serum prolactin levels and their relationship to survival in women with operable breast cancer. J Clin Epidemiol 1995;48:959–68.
48. Holtkamp W, Nagel GA, Wander HE, Rauschecker HF, von Heyden D. Hyperprolactinemia is an indicator of progressive disease and poor prognosis in advanced breast cancer. Int J Cancer 1984;34:323–8.
49. Gupta A, Dai Y, Vethanayagam RR, et al. Cyclosporin A, tacrolimus and sirolimus are potent inhibitors of the human breast cancer resistance protein (ABCG2) and reverse resistance to mitoxantrone and topotecan. Cancer Chemother Pharmacol 2006;58:374–83.
50. Qadir M, O'Loughlin KL, Fricke SM, et al. Cyclosporin A is a broad-spectrum multidrug resistance modulator. Clin Cancer Res 2005;11:2320–6.
51. Rauscher FJ III, Beerman TA, Baker RM. Characterization of auromomycin-resistant hamster cell mutants that display a multidrug resistance phenotype. Mol Pharmacol 1990;38:198–206.
52. Boesch D, Muller K, Pourtier-Manzanedo A, Loor F. Restoration of daunomycin retention in multidrug-resistant P388 cells by submicromolar concentrations of SDZ PSC 833, a nonimmunosuppressive cyclosporin derivative. Exp Cell Res 1991;196:26–32.
53. Helgason HH, Kruijtzer CM, Huitema AD, et al. Phase II and pharmacological study of oral paclitaxel (Paxoral) plus ciclosporin in anthracycline-pretreated metastatic breast cancer. Br J Cancer 2006;95:794–800.

Review

New mechanisms for PRLr action in breast cancer

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Prolactin (PRL) is a pleiotrophic hormone that contributes to the growth of normal and malignant breast tissues. PRL signals through its receptor (PRLr), a transmembrane receptor that belongs to the cytokine receptor family. The mechanism of how the PRL:PRLr interaction triggers activation of signaling networks remains enigmatic. This review examines the effect of ligand binding on PRLr and the processes that initiate receptor-associated signaling. Evidence for PRLr predimerization in the absence of ligand and the actions of the prolyl isomerase cyclophilin A in ligand-induced activation of PRLr-associated Jak2 kinase are discussed. These studies reveal that ligand-induced conformational change of the PRLr complex is necessary for its function and open avenues for therapies to inhibit PRLr action in breast cancer.

Role of PRL in the normal and malignant breast

Prolactin (PRL) was originally identified as a neuroendocrine hormone of pituitary origin. Although the pleiotropic actions of PRL are recognized, its role in regulating growth and differentiation of mammary tissues is better understood [1]. These effects are mediated by the interaction of PRL with prolactin receptor (PRLr), a member of the cytokine receptor superfamily [2]. PRL synthesis is not limited to the hypophysis; numerous extra-pituitary sites of PRL expression (including the breast, decidua and T-lymphocytes) have been identified [3]. In fact, PRL of the mammary gland has been shown to stimulate proliferation and survival [4], motility [5] and terminal maturation of normal mammary tissues [6,7]. Several lines of evidence have also indicated that PRL acts as an autocrine, paracrine and endocrine progression factor for mammary carcinoma *in vitro* and *in vivo* in rodents and humans [2,8]. These data include recent epidemiologic studies indicating that postmenopausal women with 'high-normal' levels of PRL are at increased risk of breast cancer [9]. These findings are bolstered by observations that transgenic mice overexpressing PRL at either the endocrine or the autocrine/paracrine levels develop both estrogen receptor (ER)+ and ER− well-differentiated mammary carcinomas within the first 12–18 months of life [2,10]. These observations have led some to consider PRL as a relevant therapeutic target in breast cancer, requiring a pharmacologic agent that can act at both local and systemic levels.

PRLr structure and signaling

The PRLr consists of an extracellular domain (ECD) required for ligand binding, a transmembrane domain (TM), and an intracellular domain (ICD) containing a region of membrane-proximal homology to other cytokine receptors (e.g. the Box 1/Variable Box/Box 2/X Box and its unique C-terminal tail) [2]. The hPRLr exists as seven recognized isoforms, which have different signaling properties [2] (Box 1).

Ligand binding to the long and, presumably, the ΔS1 isoform (these two being the most functional from a signaling perspective) results in the rapid phosphorylation of the ICD of the PRLr and activation of PRLr-associated signaling cascades such as the Jak2–Stat5 [11], Grb2–Sos–Ras–Raf–MEK–MAPK [3] and Nek3–Vav2–Rac1 [12,13] pathways. These signaling events induce several PRL-responsive genes such as those involved in cell proliferation (i.e. cyclin D1 and cytokine-inducible SH2-containing protein [CISH]) and in the differentiated mammary phenotype (i.e. β-casein) [14,15]. From garnered kinetic and knockout data [16,17], the Jak2 tyrosine kinase represents the most proximal kinase activated after ligand binding to the PRLr (Figure 1). The PRLr–Jak2 complex requires the membrane-proximal proline-rich Box 1 motif of the PRLr. Deletion of this Box 1 motif or replacement of the Box 1's C-terminal proline residue (with leucine) abrogates PRLr function [18]. In addition, the tyrosine residues in the C-terminal tail of PRLr contribute to the engagement and phosphorylation of Stat 5 by Jak2 [19]. Other structural domains within the PRLr (including the V Box, Box 2 and X Box) are largely uncharacterized and their signaling functions are not currently known (Box 1).

Jak2 structure, signaling and function

Jak2 is a member of the larger Jak2 family of tyrosine kinases that consists of four members that demonstrate considerable homology, delineated as Jak homology, or 'JH', domains. From the C terminus, this includes a kinase motif (JH1), a regulatory pseudo-kinase domain (JH2), a SH2 motif (JH3) and a band-four point one, ezrin, radixin, moesin homology domain (FERM) protein-interaction domain (JH4–7). Studies with growth hormone receptor (GHr) revealed that the N-terminal region of the FERM domain of Jak2 is necessary for GHr interaction [20]. After prolactin stimulation, Jak2 is rapidly activated (within 30–60 s) through trans- and/or auto-phosphorylation of two constitutively associated Jak2 molecules, revealing an essential proximate role for Jak2 in PRLr-mediated

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Box 1. PRLr isoforms

The long hPRLr was the first human PRLr isoform identified with the longest sequence and is a classic type I single-pass cell-membrane receptor that consists of an extracellular domain (ECD), a transmembrane (TM) domain and an intracellular domain (ICD), as seen in Figure I. The intermediate and ΔS1 hPRLr isoforms are generated from alternative splicing, resulting in deletion of a considerable portion of the ICD or deletion of a portion of the ECD, respectively. Two short forms of the hPRLr, known as S1a and S1b, are also generated via alternative splicing. The PRL-binding protein (PRLBP) represents the freely circulating ECD of the hPRLr and arises from a proteolytic event rather than a splicing event [52]. In addition, an antibody specific for the PRLr ICD identifies a PRLr that consists of the TM domain and the full-length ICD, an isoform termed the TM–ICD [36]. This fragment is perhaps generated upon the proteolytic cleavage that releases the PRLBP.

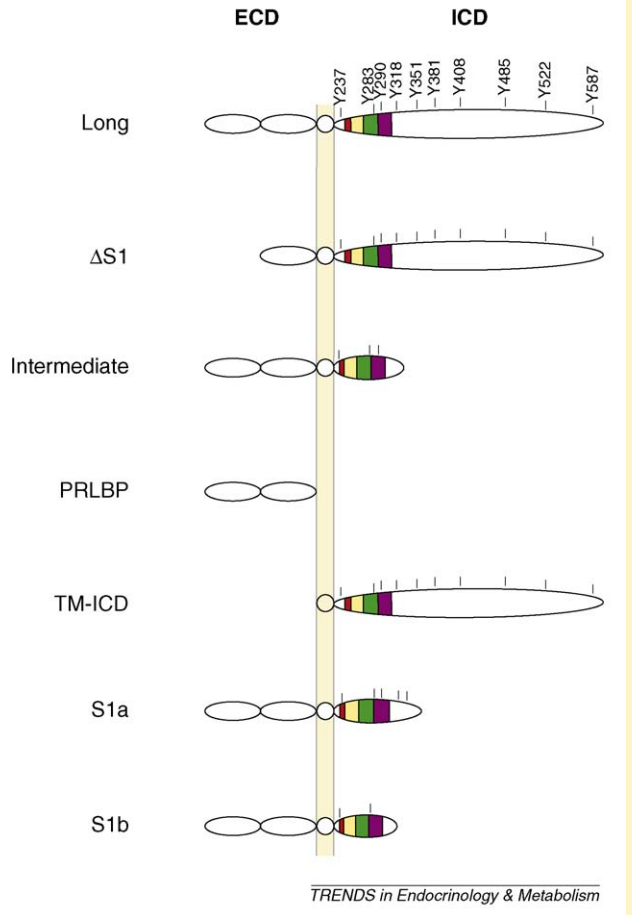

Figure I. Schema of the seven known human prolactin receptor (PRLr) isoforms. Extracellular and intracellular domains are indicated by 'ECD' and 'ICD', respectively, and the single transmembrane domain is represented by the small circle within the membrane. Designations within the ICD represent the known tyrosine residues in the PRLr, and the colored boxes represent the Box 1, V-Box, Box 2 and X-box (left to right).

signaling (Figure 1). Indeed, loss of Jak2 activity results in ablation of PRL-induced Stat5 phosphorylation and downstream gene expression [14]. Jak2 activity is necessary for the *in vitro* [21] and *in vivo* [16] growth and differentiation of mammary cells and tissues. Indeed, actions of Jak2 are not just necessary for PRLr and GHr action; they might also contribute to cross-talk between these receptors and erbB2 in breast tissues [22]. The ligand-induced mechanism that triggers the trans- and/or auto-phosphorylation of Jak2, however, has remained unresolved, not only for PRLr but also for other cytokine receptors, including GHr.

Box 2. Role of CypA in cancer

Several lines of evidence suggest that CypA might contribute to the pathobiology of human malignancy [53]. First, CypA is overexpressed in a large number of primary lung cancers [54], in human pancreatic cancer cells [55] and in oral squamous cancer cells [56]. Second, CypA overexpression in lung cancer cells enhances tumor growth *in vivo*, and stable suppression of CypA diminishes tumor growth *in vivo* [57]. Third, recent studies have demonstrated that overexpression of CypA in a variety of cancer cells confers resistance to oxidative stress-induced cell apoptosis *in vitro* [58]. Finally, significant epidemiologic data have implicated a role for cyclophilins in the pathogenesis of breast cancer. In a case cohort of 25 000 women who received CsA as therapy for renal and cardiac allografts, a significant increase in malignancies were noted at various sites, including skin, lymph tissues, kidney and oropharynx. However, a reduction in the incidence of breast cancer of up to 50% in the CsA-treated group was noted over a ten-year follow-up period. Indeed, in the first year of therapy, the CsA-treated cohort demonstrated a 90% reduction in the incidence of breast cancer [51]. Taken together, these data suggest that CypA might play an important part in tumorigenesis at multiple sites within the body.

Ligand-independent dimerization of the PRLr

The accepted view has been that the initial event in PRL signaling is the binding of one PRL molecule to two cell-surface PRLr monomers, inducing the dimerization and subsequent activation of the receptors (Figure 1a). This model was based on studies with GHr and other homologous receptors. Early studies used the bacterially expressed extracellular domain of the GHr, referred to as growth-hormone-binding protein, to determine the nature of its *in vitro* interaction with GH [23]. This classic study, however, was limited in the utilization of high concentrations of the ECD and because it did not examine the *in vivo* structural biology. Subsequent studies suggested that full-length GHr predimerization occurred *in vivo* in the absence of ligand [24,25] (Figure 1b). Indeed, Gent *et al.* [26] confirmed predimerization of GHr, demonstrating that this process occurred in the endoplasmic reticulum via the transmembrane domain, a process facilitated by the ECD [27]. Recent studies employing fluorescence resonance energy transfer (FRET), however, suggest that ECD facilitation of predimerization might not be a prerequisite [28].

Predimerization of the GHr, however, is insufficient to trigger signaling in the absence of ligand, suggesting that the function of predimerization is to hold the receptor in a 'ready' position until addition of ligand induces necessary conformational changes for activation of Jak2 kinase and subsequent signaling. Conformational changes in the ECD can lead to activation of the ICD, as evidenced by a study showing that a monoclonal antibody against the GHr ECD induces GH-independent signaling [29]. More detailed analysis suggested that GH activates a preformed dimer by transmission of rotational torque through the TM, resulting in rotation of the GHr ICDs [28]. However, this study relied exclusively on overexpressed, mutant GHrs, and these experiments were not entirely consistent with the degree of rotation predicted for GHr activation.

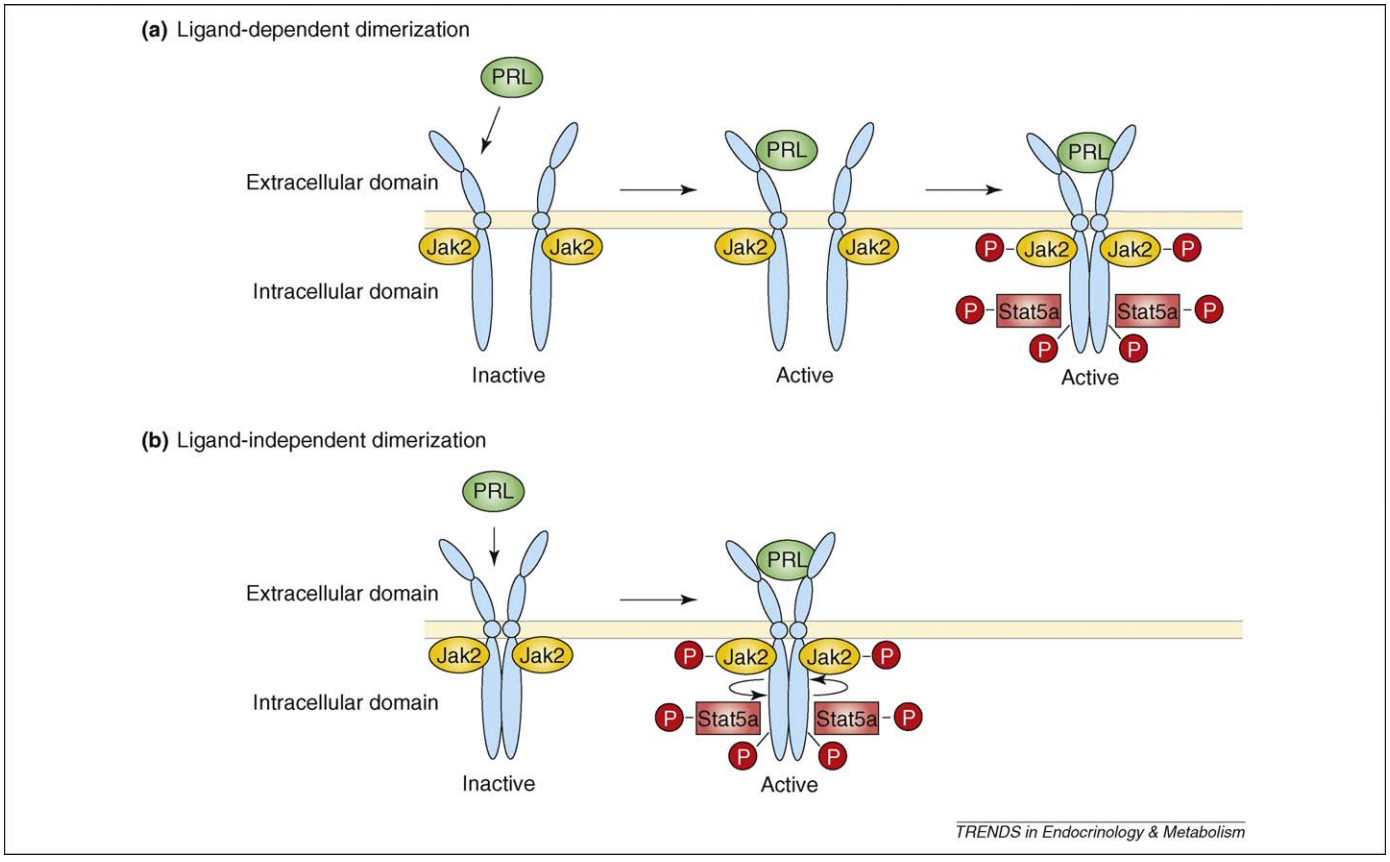

Figure 1. Ligand-dependent and -independent dimerization of the prolactin receptor (PRLr). **(a)** Ligand-dependent dimerization model. PRLr is in monomeric form at the cell membrane. One molecule of PRL first binds to one PRLr monomer via binding site 1, and this 1:1 complex then recruits the second PRLr via binding site 2. The dimerization of the two PRLrs leads to activating changes in the intracellular domain, leading to PRL signal transduction, such as phosphorylation of the Janus kinase 2 (Jak2) kinase, phosphorylation of the PRLr, and the recruitment and phosphorylation of signal transducer and activator of transcription 5a (Stat5a). **(b)** Ligand-independent model. PRLr exists in dimeric form at the cell membrane in the absence of ligand, and this interaction involves the transmembrane domain. The receptors are held in an inactive form until binding of PRL to this preformed complex induces activating changes in the intracellular domain, leading to phosphorylation of the Jak2 kinase, phosphorylation of the PRLr, and recruitment and phosphorylation of Stat5a.

Similar data have been reported for the erythropoietin receptor (EPOr). Its transmembrane domain has a major role in ligand-independent dimerization [30]. The proposed mechanism of EPOr activation upon ligand binding is via a scissor-like movement of the ICD [31], which is slightly different to the rotational torque mechanism proposed for liganded GHr [28]. This indicates that although these homologous receptors predimerize in the absence of ligand, different mechanisms might account for their ligand-induced activation.

Few studies have looked at ligand-independent dimerization of the PRLr (Figure 1b). One report demonstrated that recombinant soluble PRLr-ECD (PRLBP) does not predimerize but rather forms an unstable, transient homodimer upon PRL binding [32]. Furthermore, full-length (i.e. TM-domain-containing PRLr) failed to show FRET in the absence of PRL [33], whereas another study demonstrated lack of a bioluminescence resonance energy transfer (BRET) signal between homo- and heterodimers of long and the two short PRLr isoforms in the absence of ligand [34]. However, a recent article demonstrated ligand- independent hetero- and homodimerization of the long and two short PRLr isoforms using co-immunoprecipitation and BRET [35]. Because the discordant Qazi *et al.* [35] and Tan *et al.* [34] studies both investigated the homo- and hetero- dimerization of the long and two short isoforms using BRET analysis in the same cell line, the dissimilar results might be due to differences in the nature of the constructs used by these two laboratories and/or the insensitive plate-based FRET and BRET approaches used.

Recently, our laboratory used biochemical approaches to demonstrate ligand-independent dimerization of transfected and endogenous PRLr [36]. Co-immunoprecipitation of endogenous PRLr isoforms showed that dimerization of the hPRLr occurs at physiologic concentrations and is not an artifact of transfection. In addition, transfected epitope-tagged isoforms of PRLr demonstrated that long, intermediate and ΔS1 PRLrs form ligand-independent homo- and heterodimers. PRLr deletion constructs revealed an important role for the TM domain in ligand-independent PRLr dimerization. Although interactions between the ECD and ICD in the dimeric pair do not seem to be necessary for dimerization, interactions between these domains do seem to strengthen this dimerization. Therefore, it seems the TM domain is sufficient for ligand-independent dimerization and that this interaction is strengthened by the ECD and ICD. These data are consistent with a recent study demonstrating that a mutation of a single cysteine residue in the TM domain inhibits ligand-independent dimerization by ~30% [35].

Taken together, these data make a case for ligand-independent dimerization of cytokine receptors, including

the PRLr. Rather than induce dimerization of monomeric receptors, the addition of ligand seems to bind a preformed dimer and initiate conformational changes that lead to activation of receptor ICDs and their associated kinases (Figure 1). Given the rapid activation of Jak2 kinases within 30 s of ligand stimulation, predimerization of the cytokine receptors makes teleological sense; the delay monomeric receptors would encounter secondary to lateral diffusion through the cell membrane would result in a considerable delay in ligand-stimulated receptor dimerization and signaling. With the dimerization status of the PRLr largely resolved, the conundrum now is determining what enables the receptor to alter its conformation after ligand binding, such that its associated kinases become activated.

Peptidyl prolyl isomerase function in signal transduction

Peptidyl-prolyl isomerases (PPI) are a superfamily of enzymes that catalyze the *cis-trans* interconversion of imide bonds of proline residues. The first identified member of the cyclophilin family of PPI, cyclophilin A (CypA) was thought initially to assist in nascent protein folding *in vivo*. CypA was also identified as a cytosolic receptor of the immunosuppressive drug cyclosporine A (CsA) [37–39].

Several lines of evidence recently demonstrated that PPIs function to modulate protein signal transduction, using their PPI activity to 'switch' recognized signaling proteins into either an active or an inactive conformer [40]. One such example is the regulation of the transforming growth factor-β-receptor (TGFβR) by the PPI, FKBP12. Engagement of FKBP12 by the proline-rich sequence of the intracellular domain of TGFβR results in downregulation of ligand-stimulated receptor transphosphorylation and signaling [41,42]. Other studies revealed that CypA modulates the tyrosine kinase activity of the Tec-family member interleukin-2 inducible T-cell kinase by inducing a conformational switch within this kinase's SH2 domain [43]. In addition to their regulation of cell surface receptors, PPIs impact cell signaling by interacting with transcription factors such as c-Myb [44], IRF4 [45] and Stat5 [46,47].

Role of cyclophilins and prolyl isomerization in PRLr function

Initial studies revealed an important role for cyclophilin family members in regulating PRLr-mediated signaling. Two members of the cyclophilin family, CypB and CypA, have been found to associate with and modulate the function of Stat5 and Jak2, respectively. Triggered by PRL, CypB potentiates Stat5 function by inducing the release of the Stat repressor, PIAS3 [46,47], resulting in enhanced PRL-induced gene expression, proliferation and cell motility and increasing expression of estrogen, progesterone, growth hormone and prolactin receptors [48]. By contrast, CypA constitutively associates with PRLr and Jak2 [49], resulting in enhanced Jak2 and Stat5 activity. Cyclophilins then might function as both proximal and distal signaling switches during PRLr transduction, and pharmacologic blockade of PPI activity of CypA and/or B might downregulate PRLr signaling and function(s) in breast cancer.

To further explore the role of CypA in the proximate activation of PRLr, a multi-pronged approach was utilized that included overexpression of wild-type CypA, knockdown of CypA through small interfering RNA (siRNA), overexpression of a PPI-inactive mutant CypA, a point-mutant of PRLr that is incapable of binding CypA and the use of the cyclophilin PPI inhibitor CsA [50]. Consistent with its role as a signaling switch, overexpression of wild-type CypA resulted in significant increases in Stat5-driven gene expression from luciferase reporters consisting of singular (luteinizing hormone response element) and composite (CISH and cyclin D1) response elements, as well as the endogenous expression of both the Stat5-responsive CISH and cyclin D1 genes at the mRNA and protein levels. Conversely, 50% reductions in CypA levels mediated by CypA siRNA resulted in 2–3-fold reductions in reporter and endogenous gene expression. Overexpression of a PPI-defective form of CypA was also found to decrease PRLr/Jak2 signaling significantly. Finally, to prove that the actions of CypA associated with the PRLr were directly responsible for the activation of Jak2, the binding site for CypA on the PRLr was pinpointed through targeted mutagenesis. These studies revealed that a PRLr X-box proline residue (P334) contributed to the engagement of CypA. Replacement mutation at this site, while not altering Jak2 association, markedly decreased PRLr–CypA association and resulted in a marked reduction of Jak2 and Stat5 phosphorylation and PRL-induced gene expression. Given that T47D cells are known to express high levels of endogenous PRLr, these findings were notable and suggested that the P334A mutant functions as a dominant-negative receptor, perhaps through its heterodimerization with the wild-type receptor. When assessed with the data presented above, these findings revealed that direct action of the CypA on PRLr is required for effective PRL-induced signaling, a process that is inhibited by the P334A PRLr mutant.

CsA inhibits *in vitro* PRL-induced signaling and function in breast cancer cells

Given the notable effects of CypA on PRLr signaling, it was hypothesized that the inhibitor of cyclophilin prolyl isomerase activity, CsA, could have marked effects on PRLr-mediated signaling. Furthermore, by acting on PRLr and other cyclophilin-dependent receptors and signaling pathways, it was also hypothesized that CsA could alter the growth and progression of human breast cancer [50] (Box 2). To that end, treatment of a spectrum of ER-positive and ER-negative (and Her2+) breast cancer cell lines with CsA resulted in a significant dose-dependent inhibition of cell growth, with low-intermediate doses (1–10 μg CsA/ml) inhibiting proliferation and higher doses (30 μg CsA/ml and above) inducing cell death [50]. CsA treatment also decreased breast cancer cell colony number and colony size and inhibited cell migration and invasion [50]. CsA pretreatment of breast cancer cells resulted in a near-complete inhibition of Jak2/Stat5 phosphorylation and notable reductions in AKT and ERK activation [50]. These reductions correlated with significant decreases in PRL-induced gene expression at both the reporter and the endogenous gene level. Interestingly, although CsA had

no effect on the PRLr–Jak2 interaction, it completely abolished the interaction of CypA with the PRLr. The inhibition of receptor signaling by CsA was found to be specific, in that although PRLr- and GHr-associated signaling were reduced by CsA, no inhibition was noted for either the insulin or the epidermal growth factor receptors [50]. Taken together, these data show that CsA inhibits many of the malignant properties of breast cancer cells *in vitro*, and perhaps this inhibition could be translated into pre-clinical testing.

Potential of CsA for treatment of breast cancer

The ability of CsA to inhibit growth, motility, invasion and soft agar colony formation of human breast cancer cells suggests CsA as a potential breast cancer target [50]. In fact, female allograft patients receiving CsA for immunosuppression show a 90% decrease in incidence of breast cancer [51]. Xenograft studies in mice showed that CsA therapy markedly increased central tumor necrosis, particularly in ER+ tumors [50]. Perhaps most remarkably, not a single metastasis to lymph nodes, brain or visceral organs was noted in CsA-treated mice [50]. Indeed, in experimental groups receiving varied doses of CsA (some as low as 10 mg/kg/day), no metastases were noted (n = 150), whereas nearly 50% of the control mice demonstrated metastasis. Further analyses revealed that CsA therapy inhibited the *in vivo* association of CypA with PRLr and markedly reduced the PRL-responsive expression of cyclin D1. These highly significant results have several implications. First, they support an *in vivo* biologic role for CypA and its PPI activity. Second, they raise interesting questions regarding the function of CypA in supporting the metastatic process and potentially central tumor angiogenesis. Third, they indicate that the effects of CsA are direct and not a consequence of immunosuppressive actions, and fourth, they raise the distinct possibility that the FDA-approved drug, CsA, or analogs thereof might be useful in the treatment of human breast cancer. The pharmacokinetics of CsA with respect to its immunosuppressive actions and toxic side-effects are well understood and have been manageable in the clinical setting, a situation analogous to many of the effects clinically observed with the chemotherapeutic agents currently used to treat breast cancer. Thus, the actions of cyclophilins not only shed light on the mechanisms of proximal receptor-based signaling but also might provide considerable translational opportunities for the development of novel PPI-inhibitory therapies directed against human breast cancer.

Concluding remarks

The studies described in this review have altered our vision of how PRLr and GHr signaling is mediated. The conventional view of ligand-induced PRLr dimerization resulting in receptor-associated signaling was largely based on the artifice of *in vitro* studies using only the extracellular domains of these receptors. Subsequent analysis using full-length receptors in their *in vivo* setting has revealed an entirely different picture: that PRLr and GHr are pre-

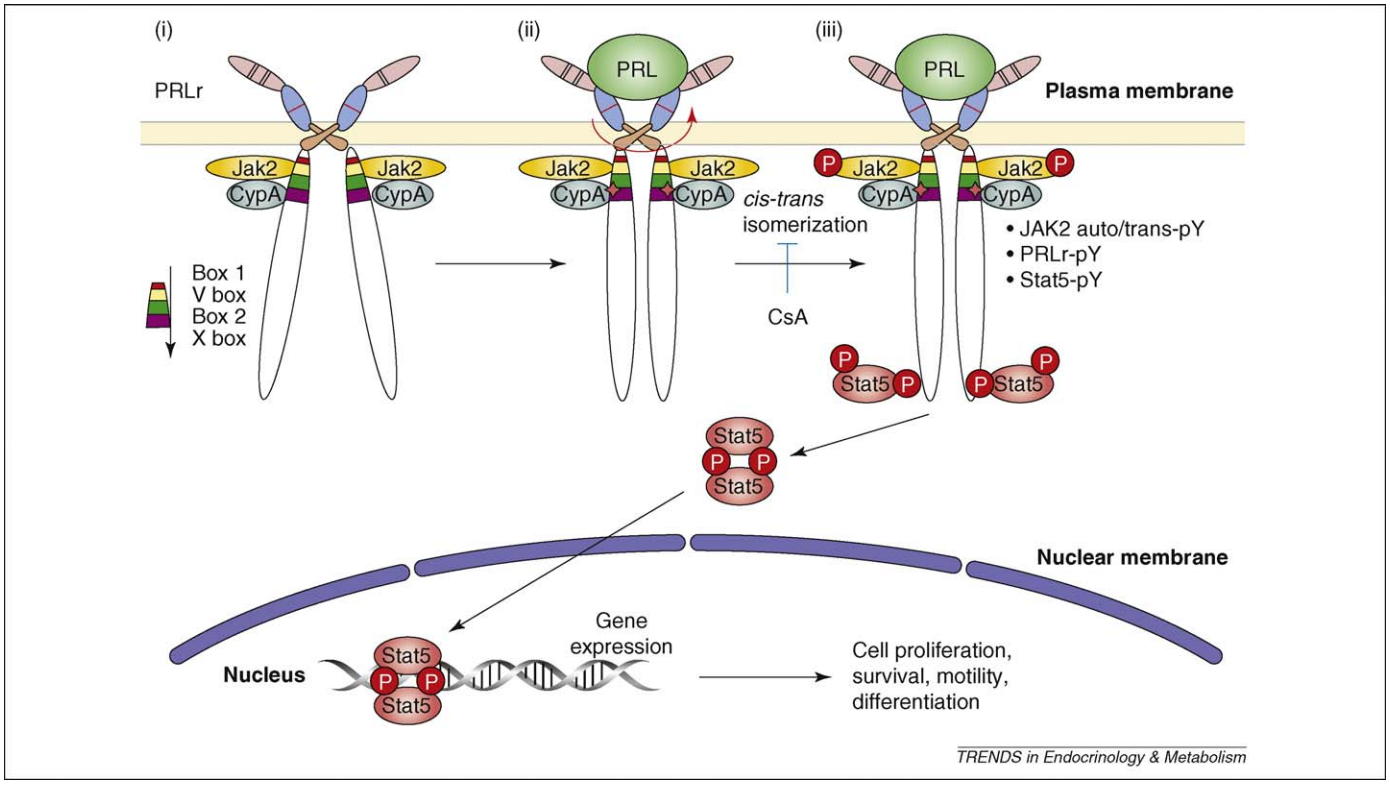

Figure 2. Regulation of the prolactin receptor/Janus kinase 2 (PRLr/Jak2) complex by PRLr-associated cyclophilin A (CypA). (i) CypA is constitutively associated with PRLr and Jak2 during unstimulated conditions. (ii) Upon binding of PRL to PRLr, CypA positively regulates Jak2 activity by exerting its isomerase activity, (iii) presumably through its switching function of a cis-trans peptidyl prolyl isomerase. Ablation of CypA PPI activity by cyclosporine A (CsA) and other approaches inhibits PRL signaling and might be a novel therapeutic strategy in the treatment of human breast cancer. The red arrow in (ii) denotes *cis-trans* interconversion of proline 334 in the X-box motif of the PRLr; the red circle containing P indicates phosphorylation of JAK2 kinase, PRLr, and signal transducer and activator of transcription 5a (Stat5a).

dimerized and that the activation of receptor-associated Jak2 kinases occurs as a consequence of conformational change in the receptors, in part mediated by prolyl isomerases, such as CypA (Figure 2). These findings raise several questions and point to future research directions. For example, regarding the ligand-independent dimerization of the PRLr, what precise domains and/or residues are involved in PRLr predimerization? What would be the effects on signaling if these domains were disrupted? In terms of the actions of cyclophilins and CsA, what are the dynamic changes within the PRLr induced by cyclophilin after ligand binding? How does CsA stimulate tumorigenesis at multiple sites in the body and yet inhibit the progression of breast cancer? Can CypA be targeted in a specific manner, such that the immunosuppressive and non-specific effects of CsA can be avoided? Is continuous therapy with CsA efficacious at preventing or inhibiting the progression of breast cancer in patients? The challenges posed by these questions present an opportunity to examine the fundamental mechanisms of proximal cytokine receptor-based signaling and translate them into novel therapies directed against disease states dependent upon the actions of prolyl isomerases.

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References

1 Bachelot, A. and Binart, N. (2007) Reproductive role of prolactin. *Reproduction* 133, 361–369
2 Clevenger, C.V. *et al.* (2003) The role of prolactin in mammary carcinoma. *Endocr. Rev.* 24, 1–27
3 Harris, J. *et al.* (2004) Prolactin and the prolactin receptor: new targets of an old hormone. *Ann. Med.* 36, 414–425
4 Perks, C.M. *et al.* (2004) Prolactin acts as a potent survival factor for human breast cancer cell lines. *Br. J. Cancer* 91, 305–311
5 Maus, M.V. *et al.* (1999) Prolactin as a chemoattractant for human breast carcinoma. *Endocrinology* 140, 5447–5450
6 Ormandy, C.J. *et al.* (1997) Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev.* 11, 167–178
7 Horseman, N.D. *et al.* (1997) Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. *EMBO J.* 16, 6926–6935
8 Graichen, R. *et al.* (2003) The growth hormone-binding protein is a location-dependent cytokine receptor transcriptional enhancer. *J. Biol. Chem.* 278, 6346–6354
9 Hankinson, S.E. *et al.* (1999) Plasma prolactin levels and subsequent risk of breast cancer in postmenopausal women. *J. Natl. Cancer Inst.* 91, 629–634
10 Wennbo, H. *et al.* (1997) Activation of the prolactin receptor but not the growth hormone receptor is important for induction of mammary tumors in transgenic mice. *J. Clin. Invest.* 100, 2744–2751
11 Clevenger, C.V. (2004) Role of Stat family transcription factors in human breast cancer. *Am. J. Pathol.* 165, 1449–1460
12 Miller, S.L. *et al.* (2004) Novel association of Vav2 and Nek3 modulates signaling through the human prolactin receptor. *Mol. Endocrinol.* 19, 939–949
13 Miller, S.L. *et al.* (2007) Nek3 kinase regulates prolactin-mediated cytoskeletal reorganization and motility of breast cancer cells. *Oncogene* 26, 4668–4678
14 Brockman, J.L. *et al.* (2002) PRL activates the cyclin D1 promoter via the Jak2/Stat pathway. *Mol. Endocrinol.* 16, 774–784
15 Fang, F. *et al.* (2008) Quantification of PRL/Stat5 signaling with a novel pGL4-CISH reporter. *BMC Biotechnol.* 8, 1–11
16 Sakamoto, K. *et al.* (2007) The Janus kinase 2 (Jak2) is required for expression and nuclear accumulation of Cyclin D1 in proliferating mammary epithelial cells. *Mol. Endocrinol.* 21, 2218–2232
17 Wagner, K-U. *et al.* (2004) Impaired alveologenesis and maintenance of secretory mammary epithelial cells in Jak2 conditional knockout mice. *Mol. Cell. Biol.* 24, 5510–5520
18 Pezet, A. *et al.* (1997) The last proline of box 1 is essential for association with JAK2 and functional activation of the prolactin receptor. *Mol. Cell. Endocrinol.* 129, 199–208
19 Pezet, A. *et al.* (1997) Tyrosine docking sites of the rat prolactin receptor required for association and activation Stat5. *J. Biol. Chem.* 272, 25043–25050
20 He, K. *et al.* (2003) Janus kinase 2 determinants for growth hormone receptor association, surface assembly, and signaling. *Mol. Endocrinol.* 17, 2211–2227
21 Xie, J. *et al.* (2002) Role of tyrosine kinase Jak2 in prolactin-induced differentiation and growth of mammary epithelial cells. *J. Biol. Chem.* 277, 14020–14030
22 Yamauchi, T. *et al.* (2000) Constitutive tyrosine phosphorylation of ErbB-2 via Jak2 by autocrine secretion of prolactin in human breast cancer. *J. Biol. Chem.* 275, 33937–33944
23 Waters, M.J. *et al.* (2006) New insights into growth hormone action. *J. Mol. Endocrinol.* 36, 1–7
24 Harding, P. *et al.* (1996) Growth hormone and a GH antogonist promote GH receptor dimerization and internalization. *Bioessays* 271, 6708–6712
25 Ross, R.J.M. *et al.* (2001) Binding and functional studies with the growth hormone receptor antagonist B2036-PEG (Pegvisomant), reveal effects of pegylation and evidence that it binds to a receptor dimer. *J. Clin. Endocrinol. Metab.* 86, 1716–1723
26 Gent, J. *et al.* (2002) Ligand-independent growth hormone receptor dimerization occurs in the endoplasmic reticulum and is required for ubiquitin system-dependent endocytosis. *Proc. Natl. Acad. Sci. U. S. A.* 99, 9858–9863
27 Yang, N. *et al.* (2007) Role of growth hormone (GH) receptor transmembrane domain in receptor predimerization and GH-induced activation. *Mol. Endocrinol.* 21, 1642–1655
28 Brown, R.J. *et al.* (2005) Model for growth hormone receptor activation based on subunit rotation within a receptor dimer. *Nat. Struct. Mol. Biol.* 12, 814–821
29 Mellado, M. *et al.* (1997) Conformational changes required in the human growth hormone receptor for growth hormone signaling. *J. Biol. Chem.* 272, 9189–9196
30 Constantinescu, S.N. *et al.* (2001) The erythropoietin receptor cytosolic juxtamembrane domain contains an essential, precisely oriented, hydrophobic motif. *Mol. Cell* 7, 377–385
31 Seubert, N. *et al.* (2003) Active and inactive orientations of the transmembrane and cytosolic domains of the erythropoietin receptor dimer. *Mol. Cell* 12, 1239–1250
32 Gertler, A. *et al.* (1996) Real-time measurements of the interactions between lactogenic hormones and prolactin-receptor extracellular domains from several species support the model hormone-induced transient receptor dimerization. *J. Biol. Chem.* 271, 24482–24491
33 Biener, E. *et al.* (2003) Ovine placental lactogen-induced heterodimerization of ovine growth hormone and prolactin receptors in living cells is demonstrated by fluorescence resonance energy transfer microscopy and leads to prolonged phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3. *Endocrinology* 144, 3532–3540
34 Tan, D. *et al.* (2005) Unmodified prolactin (PRL) and S179D Prl-initiated bioluminescence resonance energy transfer between homo - and hetero - pairs of long and short human PRL receptors in living human cells. *Mol. Endocrinol.* 19, 1291–1303
35 Qazi, A.M. *et al.* (2006) Ligand-independent homo- and hetero-dimerization of human prolactin receptor variants: inhibitory action of the short forms by hetrodimerization. *Mol. Endocrinol.* 26, 6748–6761
36 Gadd, S. and Clevenger, C.V. (2006) Ligand-independent dimerization of the human prolactin receptor: functional implications. *Mol. Endocrinol.* 20, 2734–2746

37 Colgan, J. *et al.* (2005) Cyclophilin A-deficient mice are resistant to immunosuppression by cyclosporine. *J. Immunol.* 174, 6030–6038
38 Edlich, F. and Fischer, G. (2006) Pharmacologic targeting of catalyzed protein folding: the example of peptide bond cis/trans isomerases. *Handbook of Experimental Pharmacology* 172, 359–404
39 Wang, P. and Heitman, J. (2005) The cyclophilins. *Genome Biol.* 6, 226
40 Lu, K.P. *et al.* (2007) Prolyl *cis-trans* isomerization as a molecular timer. *Nat. Chem. Biol.* 3, 619–629
41 Chen, Y-G. *et al.* (1997) Mechanisms of TGFβ receptor inhibition by FKB12. *EMBO J.* 16, 3866–3876
42 Lopez-Ilasaca, M. *et al.* (1998) Effects of FK506-binding protein 12 and FK506 on autophosphorylation of epidermal growth factor receptor. *J. Biol. Chem.* 273, 9430–9434
43 Brazin, K.N. *et al.* (2002) Regulation of the tyrosine kinase Itk by the peptidyl-prolyl isomerase cyclophilin A. *Proc. Natl. Acad. Sci. U. S. A.* 99, 1899–1904
44 Leverson, J.D. and Ness, S.A. (1998) Point mutations in v-Myb disrupt a cyclophilin-catalyzed negative regulatory mechanism. *Mol. Cell* 1, 203–211
45 Mamane, Y. *et al.* (2000) Postranslational regulation of IRF-4 activity by the immunophilin FKBP52. *Immunity* 12, 129–140
46 Rycyzyn, M.A. *et al.* (2000) Role of cyclophilin B in PRL signal transduction and nuclear retrotranslocation. *Mol. Endocrinol.* 14, 1175–1186
47 Rycyzyn, M.A. and Clevenger, C.V. (2002) The intranuclear prolactin/cyclophilin B complex as a transcriptional inducer. *Proc. Natl. Acad. Sci. U. S. A.* 99, 6790–6795
48 Fang, F. *et al.* (2009) Expression of cyclophilin B is associated with malignant progression and regulation of genes implicated in the pathogenesis of breast cancer. *Am. J. Pathol.* 174, 297–308
49 Syed, F. *et al.* (2003) A novel and functional interaction between cyclophilin (Cyp) A and the prolactin receptor. *Endocrine* 20, 83–89
50 Zheng, J. *et al.* (2008) Peptidyl-prolyl isomerase regulation of Jak2 activation and the progression of human breast cancer. *Cancer Res.* 68, 7769–7778
51 Stewart, T. *et al.* (1995) Incidence of de-novo breast cancer in women chronically immunosuppressed after organ transplantation. *Lancet* 346, 796–798
52 Kline, J.B. and Clevenger, C.V. (2001) Identification and characterization of the prolactin-binding protein (PRLBP) in human serum and milk. *J. Biol. Chem.* 276, 24760–24766
53 Yao, Q. *et al.* (2005) Roles of cyclophilins in cancers and other organ systems. *World J. Surg.* 29, 276–280
54 Howard, B.A. *et al.* (2005) Stable RNA interference-mediated suppression of cyclophilin A diminishes non-small-cell lung tumor growth *in vivo*. *Cancer Res.* 65, 8853–8860
55 Shen, J. *et al.* (2004) Protein expression profiles in pancreatic adenocarcinoma compared with normal pancreatic tissue and tissue affected by pancreatitis as detected by two-dimensional gel electrophoresis and mass spectrometry. *Cancer Res.* 64, 9018–9026
56 Rey, O. *et al.* (1999) Differential gene expression in neoplastic and human papillomavirus-immortalized oral keratinocytes. *Oncogene* 18, 827–831
57 Campa, M.J. *et al.* (2003) Protein expression profiling identifies macrophage migration inhibitory factor and cyclophilin a as potential molecular targets in non-small cell lung cancer. *Cancer Res.* 63, 1652–1656
58 Choi, K.J. *et al.* (2007) Overexpressed cyclophilin A in cancer cells renders resistance to hypoxia- and cisplatin-induced cell death. *Cancer Res.* 67, 3654–3662